Molecular epidemiology of antibiotic resistance in the commensal *Escherichia coli* of calves

Catherine M. Yates

Thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

2004

Abstract

In animals, the study of antibiotic resistance in bacteria has been focused on organisms that are pathogenic in human or animal hosts. The development of antibiotic resistance in commensal bacteria is also of concern because they may act as a reservoir of resistance genes. This thesis aimed to determine levels of resistance to veterinary and medical antibiotics in the commensal *Escherichia coli* of calves, to explore the genotypic diversity of isolates, and to study the molecular mechanism and transfer dynamics of resistance to apramycin.

The antibiotic sensitivity testing of calf faecal *E. coli*, obtained by weekly sampling, demonstrated that there was resistance to beta-lactams, cephalosporins, streptomycin, trimethoprim, chloramphenicol, tetracycline and sulphamethoxazole. These resistance phenotypes had not been selected for on antibiotic-containing media, indicating a high prevalence of the corresponding resistance determinants.

Five hundred and forty three isolates were genotyped by pulsed-field gel electrophoresis. Examination of the patterns generated by restriction with *Xba*I and analysed with BioNumerics software revealed a total of 55 different genotypes. Ampicillin resistant isolates were more diverse (24 genotypes) than apramycin or nalidixic acid resistant isolates (5 and 2 genotypes respectively). Apramycin resistance (apr^R) was conferred by three conjugative plasmids, pUK2001, pUK2002 and pUK2003, of sizes 91, 115 and 181Kb respectively. All apr^R plasmids conferred cross-resistance to the medical antibiotics tobramycin and gentamicin. Plasmids pUK2002 and pUK2003 also carried tetracycline and streptomycin resistance. Plasmid pUK2001 demonstrated very high transfer frequencies (4.12×10^{-3} during 7 hrs mating), horizontal spread to three different genotypes, and an apparent fitness advantage *in vitro*.

This thesis shows a very high prevalence of antibiotic resistance genes in the commensal faecal flora of food-producing calves. This may have significant implications for the transmission of resistance genes to human clinical bacteria.

Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated.

Catherine M. Yates

Acknowledgements

Firstly I would like to thank my supervisors Professor Mark Woolhouse, Professor Sebastian Amyes and Dr. Chris Low for their help, encouragement, and support during my Ph.D. I would also like to thank the veterinary faculty and IPRAVE (International Partnership Research Award in Veterinary Epidemiology, funded by the Wellcome Trust) for providing the funding for this work. Many thanks also to Professor Bruce Levin and members of his laboratory for introducing me to the mathematical models and competition studies described in this thesis.

The Molecular Chemotherapy Group have all been very helpful and supportive throughout this Ph.D, and I would like to thank in particular Dr. Deborah Hoyle, Dr. Leila Vali, Dr. Fiona Walsh, Dr. Ahmed Hamouda, and Dr. Tammie Schneiders for all their help and advice. Many thanks to Dr. Darren Shaw who helped with all the statistics and modelling.

Special thanks also to my wonderful friends and family who have supported, consoled, and encouraged me throughout the many ups and downs.

Publications and Presentations

Hoyle, D., Yates, C.M., Pearce, M.C., Knight, H., Woolhouse, M.E.J., Low, J.C., and Amyes, S.G.B. (2003). Epidemiology of antibiotic resistance in commensal *Escherichia coli* of cattle. Poster P612. 12th European Congress of Clinical Microbiology and Infectious Diseases, Milan, Italy.

Yates, C.M., Pearce, M.C., Woolhouse, M.E.J., and Amyes, S.G.B. (2003). Molecular epidemiology of apramycin resistant *Escherichia coli* in calves. Poster C2-1487. 43rd Annual Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, USA.

Yates, C.M., Brown, D.J., Edwards, G.F.S., and Amyes, S.G.B. (2003). Detection of TEM-52 in *Salmonella enterica* serovar Enteritidis isolated in Scotland. Poster C2-863. 43rd Annual Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, USA.

Yates, C.M., Shaw, D.J., Woolhouse, M.E.J., and Amyes, S.G.B. (2004). Fitness costs of apramycin resistance plasmids conferring cross-resistance to gentamicin and tobramycin. Poster P1466. 14th European Congress of Clinical Microbiology and Infectious Diseases, Prague, Czech Republic.

Yates, C.M., Brown, D.J., Edwards, G.F., and Amyes, S.G.B. (2004). Detection of TEM-52 in *Salmonella enterica* serovar Enteritidis isolated in Scotland. *Journal of Antimicrobial Chemotherapy* **53**, 407-408.

Yates, C.M., Pearce, M.C., Woolhouse, M.E., and Amyes, S.G. (2004). High frequency transfer and horizontal spread of apramycin resistance in calf faecal *Escherichia coli. Journal of Antimicrobial Chemotherapy* Jul 1 [Epub ahead of print]

Abbreviations

AFLP	arbitrary fragment length polymorphism
AMP	ampicillin
amp^R	ampicillin resistant
AMX	amoxycillin
APR	apramycin
apr^R	apramycin resistant
AZL	azlocillin
AZT	aztreonam
bp	base pair
cfu	colony forming units
CLV	clavulanic acid
D	donor
DM	Davis Mingioli
DNA	deoxyribonucleic acid
DNE	cephradine
ESBL	extended spectrum β-lactamase
FOX	cefoxitin
GNT	gentamicin
IST	isosensitest
Kb	kilobase pairs
LB	Lennox L broth
MIC	minimum inhibitory concentration
MLEE	multilocus enzyme electrophoresis
MM	minimal medium
nal^R	nalidixic acid resistant
P	unselected population
PCR	polymerase chain reaction
PEN	penicillin
PFGE	pulsed-field gel electrophoresis
QRDR	quinolone resistance determining region

R	recipient
RAPD	random amplified polymorphic DNA
rep-PCR	repetitive element polymerase chain reaction
RNA	ribonucleic acid
rpm	revolutions per minute
SDW	sterile distilled water
SSRL	Scottish Salmonella Reference Laboratory
STP	streptomycin
SUL	sulphamethoxazole
T	transconjugant
TAX	cefotaxime
TAZ	ceftazidime
TBX	tryptone bile X-glucuronide
TET	tetracycline
THN	cephalothin
TIZ	ceftizoxime
TOB	tobramycin
URX	cefuroxime
w/v	weight per volume

Contents

Chapter 1. Introduction

Chapter 2. Materials and reagents

Chapter 3. Antimicrobial susceptibility testing

Chapter 4. Genotyping

Chapter 5. Molecular mechanism of apramycin resistance

Chapter 6. The study of plasmid dynamics

Chapter 7. Competitive fitness costs of apramycin resistance plasmids

Chapter 8. The search for extended-spectrum beta-lactamases

List of Tables

List of Figures

Chapter 1. Introduction

Since the introduction of antibiotics to human medicine in the 1940s, and later to animal husbandry and veterinary medicine, microbial resistance to these drugs has been on the increase. The massive and often inappropriate use of antimicrobials in human and veterinary medicine, in agriculture and aquaculture, combined with the dramatic reduction in the number of new antimicrobial agents coming onto the market has led to a growing resistance problem. This complex and global phenomenon is threatening the successful management of human infectious disease.

This introductory chapter will take the form of six sections. Firstly, the antibiotic targets of action, the mechanisms by which bacteria may become resistant to different antibiotics, and the processes implementing the dissemination of antibiotic resistance genes will be described. Following this, information available on the ecology of *E. coli* in animals will be reviewed. Finally, the use of antibiotics in food-production animals will be described, before the literature documenting the impact of this usage is reviewed.

1.1 Antibiotic targets of action.

Antibiotics should act on targets present in bacterial cells with minimal host cell damage. The target may not be present in animal or human cells (e.g. a cell wall), or the antibiotic can only enter bacterial cells (e.g. tetracyclines), or the antibiotic binds selectively to the target in bacterial cells but not in mammalian cells (e.g. aminoglycosides). The first antibiotics in general use were the penicillins, which specifically target the synthesis of the bacterial cell wall. The resemblance of the penicillins (and other β-lactam antibiotics) to the substrate of the transpeptidase enzyme, causes the inhibition of this enzyme. This prevents the cross-linking of the peptidoglycan subunits, which are key components of the bacterial cell wall. Other targets of action of antibiotics include the inhibition of protein synthesis, of

tetrahydrofolate synthesis, and of the synthesis of nucleic acids. Representative antibiotics exhibiting these mechanisms of action are listed in Table 1.1.

Table 1.1 Summary of antibiotic classes and their targets of action.

Target	Antibiotic class	Antibiotics
Cell wall synthesis	Beta-lactams	Penicillins (ampicillin, amoxycillin) Cephalosporins (cefoxitin, cefuroxime Carbapenems (meropenem) Monobactams (aztreonam) Beta-lactamase inhibitors (clavulanic acid)
Protein synthesis	Aminoglycosides	Apramycin Streptomycin Tobramycin Gentamicin
	Macrolides	Erythromycin Azithromycin
	Tetracyclines	Tetracycline Oxytetracycline
	Chloramphenicol	Chloramphenicol
DNA synthesis	Quinolones Fluoroquinolones	Nalidixic acid Ciprofloxacin Enrofloxacin
RNA synthesis	Rifampicin	Rifampicin
Tetrahydrofolate synthesis	Sulphonamides Diaminopyrimidines	Sulphamethoxazole Trimethoprim

Adapted from Amyes *et al.*,1996.

1.2 Mechanisms of antibiotic resistance.

Bacteria may evade the action of an antibiotic by intrinsic or acquired mechanisms. The former is species specific and so determines the spectrum of activity of the drug, whereas the latter is only present in a subpopulation of a species. Specific mechanisms by which bacteria may become resistant to an antibiotic are listed in Table 1.2.

Table 1.2 Examples of mechanisms conferring resistance to specific antibiotics.

Resistance mechanism	Antibiotics	Type of resistance	Reference
Impermeability	Tetracycline	Transferable	Levy & McMurry, 1974.
Destruction	Beta-lactams	Transferable or chromosomal	Abraham & Chain, 1940.
Modification	Aminoglycosides	Transferable or chromosomal	Benveniste & Davies, 1971
Altered target	Quinolones, streptomycin	Chromosomal	Cox *et al*., 1964
Additional target	Trimethoprim	Transferable	Amyes & Smith, 1974
Hyperproduced target	Trimethoprim	Transferable	Young & Amyes, 1985

The resistance mechanisms listed in Table 1.2 are specific to a particular antibiotic, however bacteria may also be intrinsically resistant to a broad spectrum of antibiotics. The reduced number of porins on the outer membrane of *Acinetobacter baumanii* confers reduced susceptibility to a range of antibiotics, by limiting the extent of antibiotic penetration into the cell (Quale *et al*., 2003). Multiple antibiotic resistant (Mar) *E. coli* mutants have decreased susceptibility to many different classes of antibiotics (including tetracycline, ampicillin, nalidixic acid, rifampicin and chloramphenicol) (Cohen *et al*., 1993). Phage mediated deletion of genes encoding an efflux pump (*acrAB*) restored the original susceptibility of an *E. coli* Mar mutant, indicating that the AcrAB efflux pump plays a major role in the Mar phenotype (Okusu *et al*., 1996).

1.3 Mechanisms enabling the dissemination of resistance genes

Although intrinsic resistance to particular antibiotics may limit choices of antibiotics available for treatment, of greater concern is the acquisition of resistance by sensitive bacteria. This may occur by the processes of transduction, transformation and conjugation. These three processes will be described, before the importance of transposable elements (namely transposons and integrons) that make up mobile

resistance elements are discussed in the context of the dissemination of resistance.

1.3a Transduction.

Transduction is mediated by bacteriophages. As these generally have limited host ranges, Davies & Webb (1998) considered transduction less important in the dissemination of resistance genes than conjugation. However, Olsen *et al.*, (1974) have described two phage types capable of infecting any Gram-negative bacterium carrying the resistance plasmid RP1, so could theoretically transfer DNA between unrelated species. During transduction the DNA is carried within the phage particle and so is protected from degradation. This has led to suggestions that in natural habitats transduction as a mechanism of gene transfer may be as important as transformation or conjugation (Zeph *et al.*, 1988).

Jiang & Paul (1998) used a mathematical model to predict the potential for gene transduction in the marine environment. The model was based on one phage-host transduction system studied *in vitro*, and assumed that all marine phages are always infective and that no other factor (such as temperature, pH, non-specific attachment of phage to other particles) influenced transduction rates. The value of a numerical extrapolation of *in vitro* transduction frequencies is unclear because of these assumptions, in addition to the knowledge that transduction rates differ with different phage-host systems (Ichige *et al.*, 1989).

1.3b Transformation

Transformation is the intragenic recombination of chromosomal genes encoding sensitive drug targets, with related genes from other bacteria to form mosaic genes that encode resistant drug targets (Carlson *et al.*, 1983). The selection pressure provided by antibiotic usage has promoted the spread of mosaic genes encoding

penicillin resistant penicillin binding proteins (PBPs) among *Streptococcus* spp. (Dowson *et al.*, 1990) and sulphonamide resistant dihydropterate synthase (dhps) among *Neisseria* spp. (Fermer *et al.*, 1995). Tetracycline resistance, encoded by *tetB*, has also been reported to be disseminated by transformation, between *Haemophilus* spp. and *Moraxella catarhalis* (Roberts *et al.*, 1991).

Although most bacteria can be shown to be competent under certain conditions (Dubnau 1991; Smith *et al.*, 1981), natural transformation has been reported to occur in a limited number of genera (Carlson *et al.*, 1983; Catlin, 1960; Frischer *et al.*, 1990; Goldberg *et al.*, 1966; Juni & Janik, 1969; Lacks *et al.*, 1975; Mathis & Scocca, 1982; Rudin *et al.*, 1974; Wang & Taylor, 1990). Transformation has been considered less important than conjugation as a mechanism for dissemination of resistance genes (Roy 1999). This is because transformation requires the presence of DNA from genetically distinct bacteria in the immediate environment of the bacterium, a DNA uptake system, and a mechanism for the permanent incorporation of recruited DNA into the host chromosome. This is usually mediated by *recA* (Fischer & Haas, 2004; Pearce *et al.*, 1995). Although transformation appears to be rare, antibiotic usage provides an intense selective pressure on the complex microbial populations of the gastrointestinal tract. In this environment gene exchange may involve many steps between unrelated bacteria. The efficiency at which transfer occurs is not important if one considers that only a very small number of resistant bacteria are needed to survive and replicate to create clinical problems.

1.3c Conjugation

The transfer of DNA by conjugation requires direct contact between donor and recipient. The genetic elements that are self-transferable by conjugation are plasmids and transposons. Conjugative plasmids can transfer efficiently between different genera of the same Gram-stain group. Although conjugative plasmids can transfer between Gram-positive and Gram-negative bacteria they are often unable to replicate

in the new host because their host range for replication is less broad than that for transfer (Verma *et al.*, 1989). Certain plasmids are however, capable of replication in a bacterium of different Gram-stain but are non-conjugative (Gormley & Davies, 1991; Goze & Ehrlich, 1980).

Transfer of a non-conjugative plasmid may occur by the presence of a co-resident self-transmissible plasmid providing, *in trans*, a conjugative system to mobilise the co-resident non-conjugative plasmid. Replicative transposons may also enable the transfer of non-conjugative plasmids (Deneer *et al.*, 1982; Shoemaker *et al.*, 1986; Valentine *et al.*, 1988). These two mechanisms of transferring non-mobilisable plasmids may permit plasmid transfer between Gram-negative and Gram-positive bacteria (Doucet-Populaire *et al.*, 1992), and so enhance the fluidity of genetic transfer in mixed bacterial populations, such as the intestinal flora of animals and humans.

Similarities between plasmids as evidence supportive for *in vivo* transfer have been described on the basis of common resistance patterns (Smith 1975), incompatibility groups (Grant & Pittard, 1974; Levy, 1976) and plasmid molecular weight (Kruse & Sørum, 1994; Nijsten *et al.*, 1995). More convincing evidence for plasmid similarity would be provided by restriction enzyme digests of the plasmid using more than one enzyme, yielding identical banding patterns with each enzyme, because plasmids of apparently identical sizes may prove to be different after restriction analysis.

Plasmids encoding aminoglycoside 3-N-acetyltransferase (conferring apramycin and gentamicin resistance) have been found in isolates from cattle and humans (Chaslus-Dancla *et al.*, 1991). A high degree of genetic homology was detected by preparing plasmid DNA and restricting with three different restriction enzymes, thus providing good evidence for plasmid exchange between bacteria of human and animal origin. Using the same method, identical apramycin resistance plasmids have been found in *E. coli* and *S. typhimurium* isolated from calves, where prior to apramycin treatment of the animals, the plasmid had only been found in *E. coli*. (Hunter *et al.*, 1992).

Conjugative transposons are discrete DNA sequences capable of self-excision and integration into DNA, which are normally integrated into a bacterial genome (Franke & Clewell, 1981). Many multiple-antibiotic resistant *Streptococcus* and *Staphylococcus* spp. from clinical isolates have large conjugative transposons (Le Bouguenec *et al.*, 1990). Plasmids have not been identified in these strains suggesting that conjugal transposons were responsible for the spread of multiple resistance observed (Scott, 1992).

Conjugative transposons were first identified in Gram-positive cocci (Franke & Clewell, 1981; Shoemaker *et al.*, 1980), but since then, have also been found in a number of Gram-negative bacteria including *Neisseria gonorrhoeae* (Swartley *et al.*, 1993), *Haemophilus ducreyi* (Roberts, 1990), and *Bacteriodes* spp. (Nikolich *et al.*, 1994a). Conjugative transposons are thought to make a considerable contribution to the spread of resistance genes amongst *Bacteroides* and other bacteria transiently colonising the human intestine (Salyers & Shoemaker, 1996). However this opinion is based on the detection of *tetQ*, (which is most commonly located on conjugative transposons in *Bacteroides* spp. (Nikolich *et al.*, 1994b)) as an indicator of the presence of a conjugative transposon in this species (Salyers & Shoemaker, 1996). At present, conjugative transposons have not been found in *E. coli*.

1.3d Transposons and integrons

The incorporation of DNA, in the form of transposons and integrons, into mobile elements results in the evolution of resistance determinant plasmids (R plasmids) (Collis *et al.*, 1993), which may then be spread via conjugation (Antunes *et al.*, 2004; Heir *et al.*, 2004; Pezzella *et al.*, 2004).

The importance of transposons and integrons is due not only to their ability to enhance the dissemination of resistance genes, but also their ability to integrate into

the bacterial chromosome permitting the persistence of the resistance genes carried. Transposons have been implicated in the spread of tetracycline resistance in *Neisseria* spp. (Swartley *et al.*, 1993) and *Bacteroides* spp. (Nikolich *et al.*, 1994b), the spread of β-lactamase enzymes in *Proteus mirabilis*, *E. coli* (Naas *et al.*, 2003) and amongst *Salmonella* spp. (Orman *et al.*, 2002), and the spread of trimethoprim resistance (linked to streptomycin resistance in 8/12 plasmids examined) in clinical *E. coli* (Towner *et al.*, 1982). Integrons are commonly found on plasmids (Briggs & Fratamico, 1999), but recently a new gene conferring resistance to chloramphenicol and florfenicol has been described that is surrounded by two class I integrons, on the *S. typhimurium* chromosome (Arcangioli *et al.*, 1999). This has been speculated to allow persistence of resistance genes in the absence of antibiotic selection (Threlfall *et al.*, 1994). In a study of sulphonamide resistance in Enterobacteriaceae, stable integration of *sulI* and *sulII* into transposons and plasmids was deduced as the mechanism responsible for the extremely efficient dissemination of these genes in the Gram-negative bacteria studied (Rådstrom *et al.*, 1991).

1.4 Ecology of *E. coli* in farm animals

E. coli typically colonise the lower intestine of new-born mammals within hours. Smith & Crabb (1961) studied the faecal flora of healthy calves, and found large numbers of *E. coli* (approximately 10^5 cfu/g), *Clostridium welchii* and streptococci after 1 day. Colonising *E. coli* are thought to be acquired from the environment, other animals, directly from faeces, or from contaminated food or water (Maison & Richardson, 1981). After ingestion, *E. coli* may be present in the lumen of the intestine, or may persist in the intestine for longer periods of time by adhering to the intestinal mucosa (Freter *et al.*, 1983) with type 1 somatic pili (Freter, 1981). Certain strains of *E. coli* are found to persist for several weeks or months (Sears & Brownlee 1952; Hinton, 1986) before being replaced by other strains. Caugant *et al.* (1981), suggest that genetic diversity among *E. coli* strains transiently colonising the gastrointestinal tract is attributable to successive invasion by *E. coli* from

environmental sources, although in this study only 22 faecal samples were obtained over a period of eleven months.

Factors influencing *E. coli* ecology are unclear, partly because of a limited number of studies on the subject, inadequate typing or sampling protocols, and because only a tiny proportion *of E. coli* present in an animal can be studied in any detail. Diet has been found to influence *E. coli* numbers (Diez-Gonzalez *et al.*, 1998; Hovde *et al.*, 1999; Kruse *et al.*, 2003), in addition to stress, disease, composition of other intestinal flora (Hartl & Dykhuizen, 1984; Shedlofsky & Freter, 1974), animal management practices (Hinton *et al.*, 1984) and host genome (Toivanen *et al.*, 2001).

Antibiotic treatment also affects the ecology of *E. coli* in farm animals (Delsol *et al.*, 2003; Dunlop *et al.*, 1998). Resistance to antimicrobial agents is caused by the use of these agents in any dose and over any time period exerting a selective pressure on the microbial population. Those micro-organisms harbouring a mutation(s) in the drug target site, or those that have acquired novel biochemical activities conferring resistance, will survive to produce progeny bearing the same resistance determinants. The selective pressures responsible for the emergence of resistant bacteria in cattle include antibiotic usage for therapy, prophylaxis and growth promotion. There has been considerable debate over the past 40 years on whether antibiotics administered to animals can lead to the selection of resistant bacteria that, on entering the food chain, may result in human infection by these less sensitive strains. In 1969, human health concerns over the widespread use of antimicrobial agents as growth promoters precipitated the appointment of the Swann Committee. Its report (Swann committee 1969), stated that "the administration of antibiotics to farm livestock, particularly at sub-therapeutic levels, poses certain hazards to human and animal health". More precisely, that this practice had resulted in the emergence of resistant enteric bacteria whose resistance determinants were transmissible to other bacteria, and that these more resistant strains were transferable from animals to man.

A description of the use of antibiotics in agriculture now follows, after which the

potential impact this has on the agricultural industry and on public health is reviewed.

1.5 Agricultural uses of antibiotics

In the UK in 2002, 484 tonnes of active antimicrobial agents were sold for use in food animals (Veterinary Medicines Directorate, 2002). In farm animals, antibiotics are used therapeutically to treat disease (e.g. calf pneumonia and neonatal *E. coli* infections), prophylactically to prevent disease, and thirdly as growth promoting agents. Ten percent of the antibiotics used in agriculture are given therapeutically, the remainder are used as growth promoters and prophylactic agents (Chadwick & Goode, 1997).

Although the exact mechanisms of action of growth promoters is unknown, the administration of these agents to animals results in an increase in weight gain (between 3 and 5% depending on the species (Select Committee on Science and Technology, 1998). Growth promoters are thought to interact with microbial flora of the gastrointestinal tract, improving digestion, absorption and metabolism, and may suppress infectious disease and metabolic and fermentation disorders (Feighner & Dashkevicz, 1987). Animals fed growth promoters require less food and produce less waste (Gaskins *et al.*, 2002). Growth promoters have allowed savings to be made on space, labour and cost of rearing farm animals, because of the concurrent reduction in the occurrence of disease associated with stress and overcrowding. This has lead to increased intensification of farming practices albeit alongside continuing criticism that growth promoters are being used to substitute good husbandry and hygiene (Select Committee on Science and Technology, 1998).

1.6 Effects of antibiotic usage in food animals

The negative effects of antibiotic usage in animal agriculture include a reduction in the effective life of antibiotics used in animal therapy, foodstuff contamination with antibiotic residues, and the selection of resistant bacteria, both zoonotic and commensal. However, antibiotic use in food animals is required for the treatment of bacterial diseases, resulting in healthier animals and subsequent economic benefits. Reports of the negative effects of antibiotic usage in food production animals will be reviewed first, followed by the advantages of such antibiotic use.

1.6a Negative effects of antibiotic use in food animals

i) Selection of resistant bacteria

The use of antibiotics in farm animals will select for resistant bacteria (Levy, 1987), in the same way that antibiotic use in humans has selected for resistant bacteria. Antibiotics used prophylactically are usually administered to groups of animals and may occur over prolonged periods of time. Salyers (1999b) described this method of administration as being more likely to lead to the emergence of resistant strains than prophylactic use in human medicine, because of the numbers of animals involved. There appears to be no concrete evidence that this is the case.

The amount of antibiotic used and the time frame of administration are important parameters affecting the frequency at which resistant bacteria arise. Lipsitch & Levin (1997) have used mathematical models to predict the emergence of resistance during antibiotic treatment. Here they show how the probability of a resistant mutant appearing depends on the initial size of the susceptible population, cell division, mutation and net killing rates. If the killing rate of the susceptible bacteria is low compared to the rate of cell division, the likelihood of a resistant mutant appearing is

increased. The model predicts a shorter time span before the appearance of the first mutant if the killing rate is slower, consistent with observations made by others that sub-inhibitory concentrations of antibiotic may be effective in selecting for resistant mutants (Haas *et al.*, 1990; Tenney *et al.*, 1983).

The concentrations of antibiotic used for growth promotion are described as subtherapeutic (Donabedian, *et al.*, 2003a; Johnston, 1998). This term gives the impression that the concentrations used are too low to affect bacterial growth and hence select for resistant populations. Although these concentrations are ineffective for therapy, they may still exert a slight selective pressure on a bacterium, allowing it to grow and acquire new DNA or accumulate mutations increasing its resistance to the drug (Aarestrup *et al.*, 2000; Boerlin *et al.*, 2001; Emborg *et al.*, 2003).

The emergence of resistant bacteria through the use of antibiotics in food production animals may lead to treatment failures and economic problems for the individual farmer and on a wider scale, the agricultural industry. This is exemplified by an outbreak of multi-resistant *Salmonella typhimurium* that occurred in 1998 on a calf farm in Arizona. The deaths of many calves due to the outbreak resulted in the farm going out of business (Salyers, 1999b).

It has been suggested that if antibiotic selection is continuous, as is the case with the use of growth promoters, bacteria may accumulate compensatory mutations allowing maintenance of their resistance mechanisms without a concurrent reduction in fitness in the absence of the antibiotic (Salyers, 1999b). There are now a few studies reporting an *in vitro* amelioration of the cost of antibiotic resistance (Dahlberg & Chao, 2003; Lenski *et al.*, 1994; Schrag *et al.*, 1997). Bouma & Lenski (1988) demonstrated that carriage of a plasmid encoding chloramphenicol and tetracycline resistance by *E. coli* B, reduced bacterial fitness in the absence of chloramphenicol. After 500 generations of culture in the presence of chloramphenicol, the plasmid enhanced the fitness of its host even in the absence of antibiotic. Sequencing and characterisation of the nature of the genetic change would be required to validate

such a conclusion. Additionally, no controls were used to ensure that the genes encoding resistance were being expressed in each generation, as gene repression in the absence of antibiotic may be effective in reducing the cost of resistance.

If fitness costs associated with an antibiotic resistant phenotype are compensated for by adaptive mutations, why are many bacteria still sensitive to drugs introduced some 40 years ago e.g. β-lactams, trimethoprim (Livermore *et al.*, 2000) and tetracyclines (Österblad, 2000)? According to DANMAP (1998), glycopeptide resistant isolates (measured as resistance to avoparcin) from broilers have decreased from 82% in 1995 to 9% in 1998, coinciding with a Danish ban in 1995 on the use of avoparcin as a growth promoter. Similarly, avilamycin has not been used since 1998 and a decline in resistant enterococci from broilers from 79% in 1996 to 29% in 1998 has been demonstrated (DANMAP, 1998). These results are suggestive of a fitness disadvantage associated with the acquisition of a specific antibiotic resistance mechanism.

ii) Antibiotic residues contaminating foodstuffs

Antibiotics used in food animal production may remain in the animal's tissues, and following ingestion of the meat the residues may select for resistant bacteria in the intestine of the consumer (Anadon *et al.*, 1999; McCracken *et al.*, 1976). A major flaw in many of these experiments is that the meat products are usually consumed after cooking, yet raw tissue is often studied (Anadon *et al.*, 1999). Antibiotic residues in foodstuffs are likely to constitute a comparatively minor threat to public health in comparison to the massive use of antibiotics directly administered to humans both in hospitals and in the community (Corpet, 1987; Levy, 1998). Laws governing the number of days an animal should be antibiotic free prior to slaughter are also in place to protect consumers from the risk of antibiotic residues in foodstuffs.

iii) Selection of antibiotic resistant zoonotic pathogens

The potential hazard posed by the selection of zoonotic pathogens (e.g. *Salmonella* spp. *Campylobacter* spp. and *E. coli* O157:H7) follows the contamination of foodstuffs and subsequent human infection where antibiotic therapy is compromised by resistance determinants carried by the pathogen. It has proved difficult to find clear-cut results demonstrating that this kind of scenario is occurring. In addition, as gastroenteritis caused by these pathogens is generally not treated with antibiotics (indeed their use is contraindicated in the case of salmonellosis and *E. coli* O157:H7 infection), this potential hazard has been deemed unimportant (Linton, 1984).

Evidence supporting the transmission of antibiotic resistant *E. coli* from poultry to the human gut, following ingestion of cooked chicken is provided by Linton (1977b). After handling *E. coli* of poultry origin only 1 of 5 volunteers were found to be excreting resistant *E. coli*. The strain from the poultry and that recovered from the faeces of the volunteer were declared identical based on their antibiotic resistance pattern, plasmid profiles and serotyping. A greater number of volunteers and genotyping by PFGE would provide more definitive evidence.

Studies by Endtz *et al.*, (1991) on quinolone resistance in *Campylobacter* have shown an increase in the prevalence of resistant strains coinciding with the extensive use of enrofloxacin in poultry. The conclusions drawn that the resistance observed is mainly due to the use of this drug in the poultry industry, is substantiated by evidence that person to person transmission of *Campylobacter* is rare (Skirrow, 1977).

The spread of chloramphenicol resistant *Salmonella newport* from dairy farms to hamburgers resulting in human illness has been reported by Spika *et al.* (1987). A risk factor identified by Spika *et al.* (1987), for illness caused by chloramphenicol-resistant Salmonella was the use of penicillin or tetracycline in the month prior to onset. The use of these drugs may provide those bacteria harbouring a multiple

antibiotic resistance plasmid to outgrow other salmonellae ingested at the same time, resulting in an infection by the resistant strains. However there is some evidence that strains of salmonellae carrying multi-resistance plasmids may be more virulent than sensitive ones due to an increased persistence of plasmid bearing host organisms in the intestine (Timoney & Linton, 1982).

Other examples of the transmission of resistant bacteria from animals to humans include indistinguishable glycopeptide-resistant enterococci isolated from turkeys and their farmer in The Netherlands (van den Bogaard *et al.*, 1997), and highly similar ceftriaxone-resistant *Salmonella enterica* serovar Typhimurium in a child and cattle in Nebraska (Fey *et al.*, 2000). Similar to the study by Spika *et al.* (1987), in this latter report, the child had received extensive treatment with β-lactam antibiotics (a 10-day course of amoxycillin-clavulanate, prophylaxis with ampicillin-sulbactam before appendectomy, then post operative treatment with amoxycillin-clavulanate) prior to the onset of Salmonellosis. These treatments are likely to have selected for the ceftriaxone-resistant strain. One problem with these studies is that the environmental reservoir of such isolates is not examined, and so the possibility of acquisition of the resistant strain from the environment cannot be excluded.

The literature on the transmission of resistant bacteria from animals to humans is limited. This may be because resistance is not looked for in these illnesses as antibiotics are rarely used, but also because zoonoses are preventable by taking the appropriate care with food preparation.

iv) Selection of antibiotic resistant commensals

The use of antibiotics in agriculture increases resistance levels in animal commensal bacteria (Van den Bogaard & Stobberingh, 2000). The contamination of meat in abattoirs by these commensals is inevitable (Howe *et al.*, 1976b; Linton *et al.*, 1977b) but the extent to which resistant strains are transferred into the human intestine is

unknown.

To date, there appears to be only one report describing how the ingestion of resistant bacteria resulted directly in failed therapy, and this case does not imply contamination from the farm or processing. Ingestion of food contaminated with methicillin resistant *Staphylococcus aureus* (MRSA) resulted in an outbreak of MRSA (Kluytmans *et al.*, 1995). However, the index patient was severely immunocompromised and had taken oral ciprofloxacin and antacids (respectively, these two drugs may have selected for the MRSA strain and neutralised gastric acid used as a defence against infection). This kind of scenario is likely to be extremely unusual, but demonstrates food as a vector for resistant organisms.

There are a few reports stating that the antibiotic-resistant organisms causing illness in humans are different strains to those prevalent in animals. Nijsten *et al.* (1996) reported that the resistance of the faecal *E. coli* of pig farmers and their pigs are distinctly different. Similarly, urinary tract infections in women working in poultry processing plants were found to be caused by *E. coli* with different antibiotic resistant patterns to those isolated from the poultry (Parsonnet & Kass, 1987). In contrast to this, studies on vancomycin-resistant enterococci (VRE) have shown that the same ribotypes isolated from poultry were forming part of the normal bowel flora in humans (Bates *et al.*, 1994). The selection of these strains by antibiotic usage in hospitals was suggested as an explanation for the emergence of VRE in hospitals.

Whether or not one particular resistant bacterial strain is directly responsible for human illness is unimportant if one considers that if a bacterium carrying resistance determinants colonises the human gut (even just transiently), there is potential for the spread of these determinants to other intestinal flora. Teuber & Perreten (2000) have reported that the exchange of resistance genes between bacteria from uncooked meat or unpasteurized cheese, and human bowel flora (*Enterococcus* spp.) during passage through the intestine is highly probable. Again, the degree of movement of resistance genes, between bacteria colonising the human bowel, is unknown (Salyers, 1999b).

Evidence that horizontal gene transfer does occur in the intestine is provided by Nickolich *et al.*, (1994b), where *tetQ* in *Prevotella ruminicola* from animals had greater than 95% DNA similarity to *tetQ* from human colonic *Bacteroides* spp. Based on DNA sequences flanking the gene the authors suggest transfer occurred from humans to animals.

Similarly, almost identical copies (> 99% DNA sequence identity) of the *erm*G gene, encoding erythromycin resistance, have been found in *Bacillus sphaericus* and *Bacteroides* spp. These are found in soil and in the human colon respectively (Cooper *et al.*, 1996). Ingested soil bacteria may have transferred the gene, which is found on a conjugative transposon ($Tc^{r}Em^{r}$7853) to *Bacteroides* species in the human gut. It is unclear as to in which bacteria the gene is most likely to have arisen. Bacteria that colonise soil may encounter bacteriocins and antibiotics produced by other soil bacteria, but bacteria that normally colonise the human intestine may be dosed with various antibiotics, usually not intended to have an effect on commensal bowel flora such as *Bacteroides*.

This example illustrates an important flaw in retrospective studies, in that the direction of transfer is difficult to prove with absolute certainty. There are many examples of laboratory experiments demonstrating gene transfer between diverse bacteria (Doucet-Populaire *et al.*, 1992; Chaslus-Dancla *et al.*, 1986; Gordon, 1992; Hunter *et al.*, 1992). These are reviewed by Mazodier & Davies (1991) and are often used to obtain evidence of intergenic transfer of resistance determinants in nature. Intergenic transfer observed in such laboratory experiments is not conclusive evidence that this occurs in nature.

1.6b Advantages of antibiotic use in food animals

Antimicrobial chemotherapy is vital for the treatment and prevention of bacterial diseases in animals (e.g. calf pneumonia, mastitis and neonatal *E. coli* infections),

which may cause pain and distress or be potentially fatal. For bacterial diseases where vaccines are unavailable, the treatment of animals with antibiotics is necessary for both humane reasons and for economic reasons (preventing further spread of the etiological agent).

Antibiotic usage in food animals may reduce the loads of salmonellae and campylobacter shed by food animals, thereby helping to diminish the risk to humans from these zoonoses (Phillips *et al.*, 2004).

Growth promoters increase rates of bodyweight gain, but may also reduce incidences of acute pneumonia and metabolic and fermentative disorders in cattle, and improve heat tolerance and immune status (Gaskins *et al.*, 2002).

The European ban on the use of several growth promoting agents has resulted in decreases in avoparcin resistant animal enterococci (DANMAP, 2002), virginiamycin resistant enterococci in chickens (DANMAP, 2002), and human vancomycin resistant enterococci (van den Bogaard *et al.*, 2000). However, some therapeutic uses of antibiotics have increased as a direct result of this ban (Casewell *et al.*, 2003). In Denmark increases in tetracycline, penicillins, sulphonamides, macrolides and aminoglycosides have followed the ban on growth promoters (DANMAP, 2002). Similarly, increases in therapeutic use of antibiotics followed the 1986 growth promoter ban in Sweden (Wierup, 2001).

1.7 Conclusions

This review describes the targets of antibiotics and the mechanisms by which bacteria may acquire resistance against antibiotics. Once resistance has evolved in a bacterium, the genes for this resistance mechanism may be spread to other bacteria of the same or of different species, by the processes of transduction, transformation and conjugation.

Antibiotics are used in animal husbandry for the treatment of disease, prophylaxis, and growth promotion. This usage results in the selection of antibiotic resistant zoonotic and commensal bacteria.

E. coli is one of the normal microbial constituents of the gastrointestinal tract of mammals, and colonises the mammalian intestine within hours of birth. Although some factors influencing intestinal *E. coli* ecology have been characterised, there is little data available on the ecology of commensal *E. coli* of healthy food animals. The need for surveillance of antimicrobial resistance in both pathogenic and commensal bacteria has been stressed by many governmental and non-governmental bodies, and by independent scientists (Caprioli *et al.*, 2000; Jones, 2000; Masterton, 2000; O'Brien, 1997; Salyers, 1999a; World Health Organisation, 1997). Against a backdrop of debate on the risks to human health posed by antibiotic usage in animal husbandry (Phillips *et al.*, 2004), a better understanding of the ecology of commensal *E. coli* in healthy food animals, the levels of antibiotic resistance present, and the mechanisms of antibiotic resistance harboured by this population is required.

1.8 Aims of the thesis

Although there have been previous studies on antibiotic resistance, these were performed without the benefit of modern molecular techniques. The overall aim of this project is to examine the epidemiology of resistant bacteria in a cohort of animals in close geographic proximity. The more specific aims of the thesis are listed below:

- Determine the antibiotic sensitivities of calf faecal commensal *E. coli* to a range of veterinary and medical antibiotics.

- Determine the genotypic diversity of calf faecal commensal *E. coli*.

- Determine the mechanism of resistance to apramycin in the commensal *E. coli* population.

- Explore the use of a mathematical model in characterising the dynamics of resistance plasmid transfer.

- Determine whether apramycin resistance plasmids confer a competitive fitness disadvantage on a host bacterium.

- Determine whether calf faecal commensal *E. coli* harbour extended-spectrum β-lactamase enzymes.

Chapter 2. Materials and Reagents

2.1 Study animals and treatment records

Eleven beef-suckler calves on a non-organic Scottish farm, born between 14th September and the 1st of October 2001 were studied. The sampled calves were part of a cohort of 49 calves, which were kept separate from all other animals on the farm. The only treatments received by these 49 calves are shown Table 2.1. Four of the eleven calves sampled in this study received antibiotic treatment(s). These calves are highlighted in blue.

Table 2.1 Antibiotic treatment records for calves sampled in this study (blue calf Ids), and calves housed with sampled calves (black calf IDs).

			Antibacterial agent	
Calf ID	Treatment date	Disease	Generic name	Trade name
688	09/10/01	pneumonia	enrofloxacin	Baytril Max
693	21/11/01	pneumonia	tylosin	Tylan
	23/11/01	pneumonia	florfenicol	Nuflor
	05/12/01	pneumonia	florfenicol	Nuflor
697	01/10/01	navel ill	enrofloxacin	Baytril Max
	09/10/01	navel ill	tylosin	Tylan
	12/11/01	navel ill	tylosin	Tylan 200
698	01/10/01	navel ill	enrofloxacin	Baytril Max
699	09/10/01	navel ill	tylosin	Tylan
711	30/10/01	navel ill	enrofloxacin	Baytril Max
	06/11/01	scouring	amoxicillin + clavulanic acid	Synulox bolus
	09/11/01	navel ill	tylosin	Tylan200
717	20/12/01	pneumonia	oxytetracycline	Terramycin LA
719	09/11/01	navel ill	tylosin	Tylan200
720	09/11/01	navel ill	tylosin	Tylan200
722	05/12/01	pneumonia	florfenicol	Nuflor
723	05/12/01	scouring	amoxicillin + clavulanic acid	Synulox bolus
	20/12/01	pneumonia	oxytetracycline	Terramycin LA

2.2 Bacterial isolates

Rectal faecal samples were taken within 48h of birth. Calves were subsequently sampled approximately weekly until the 14th January 2002 excepting two weeks at Christmas and New Year. Samples were stored at 4°C and processed within 48h of collection. Samples were diluted 1:10 in maximum recovery diluent (Oxoid, Basingstoke, UK). Ten microlitres of each suspension was spread onto Chromocult TBX (tryptone bile X-glucuronide) agar (Merck, Darmstadt, Germany), incorporating 16mg/L ampicillin, or 8mg/L apramycin, or 8mg/L nalidixic acid (Sigma) or without antibiotics. Following overnight incubation at 44°C, glucuronidase positive *E. coli* were picked from each plate showing growth. The number of picks taken per plate differed depending on the number of colonies present, but in all cases, every discrete colony present on the plate was picked. Colonies were purified by subculturing twice on MacConkey agar (Oxoid, Basingstoke, UK), and then stored at -70°C on cryogenic beads (Mast Diagnostics, Germany). Other bacterial strains used in this study are listed in table 2.2.

Table 2.2 Characteristics and sources of bacterial strains used in this work.

Bacterial Strain	Characteristics	Source
E. coli NCTC10418	Standard strain	R. Paton (Edinburgh University)
P. aeruginosa NCTC10662	Standard strain	R. Paton (Edinburgh University)
S. aureus NCTC6571	Standard strain	R. Paton (Edinburgh University)
E. coli NCTC11560	TEM-1 positive strain	R. Paton (Edinburgh University)
E. coli 39R861	Plasmid sizing standard	Scottish Salmonella Reference Laboratory, Glasgow
E. coli V517	Plasmid sizing standard	Scottish Salmonella Reference Laboratory, Glasgow
S. enterica serovar Enteritidis	Outbreak strain	Scottish Salmonella Reference Laboratory, Glasgow
E. coli MG1655	Standard strain	A. Roe, ZAP laboratory (Edinburgh University)
E. coli MG1655kan	Kanamycin resistance in place of *lacZY* genes	A. Roe, ZAP laboratory (Edinburgh University)
E. coli J53	*Pro*$^-$ *Met*$^-$	S.G.B.Amyes, MC laboratory (Edinburgh University)
E. coli J62-2	*Pro*$^-$ *His*$^-$ *Trp*$^-$ Rifampicin resistant	S.G.B.Amyes, MC laboratory (Edinburgh University)

2.3 Bacterial identification

Glucuronidase positive *E. coli* were identified by the growth of blue colonies on TBX agar following overnight incubation at 44°C.

2.4 Antimicrobial agents

Antimicrobial agents, solvents, and supplier information is listed in table 2.3. Antibiotic stock solutions were prepared on the day of use and diluted with sterile distilled water if necessary.

Table 2.3 Antimicrobial agents, solvents and their suppliers.

Antimicrobial agent	Solvent	Supplier
Amoxyclavulanic acid	Sterile distilled water (SDW)	Smithkline Beecham
Ampicillin	SDW	Sigma-Aldrich
Apramycin	SDW	Sigma-Aldrich
Azithromycin	Ethanol and SDW	Pfizer Ltd
Azlocillin	$NaHCO_3$	Sigma-Aldrich
Aztreonam	SDW	Sigma-Aldrich
Cefotaxime	SDW	Sigma-Aldrich
Cefoxitin	SDW	Sigma-Aldrich
Cephradine	SDW	Sigma-Aldrich
Ceftazidime	SDW	Glaxo laboratories
Ceftizoxime	SDW	Fujisaun Pharmaceuticals
Cefuroxime	SDW	Sigma-Aldrich
Cephalothin	SDW	Eli Lilly Phamaceuticals
Chloramphenicol	Ethanol and SDW	Sigma-Aldrich
Ciprofloxacin	SDW	Bayer plc
Enrofloxacin	SDW	Fluka
Gentamicin	SDW	Sigma-Aldrich
Nalidixic acid	SDW	Sigma-Aldrich
Penicillin	SDW	Sigma-Aldrich
Rifampicin	Ethanol and SDW	Marion Merrell Ltd.
Streptomycin	SDW	Sigma-Aldrich
Sulphamethoxazole	0.1M NaOH and SDW	Sigma-Aldrich
Tetracycline	SDW	Sigma-Aldrich
Tobramycin	SDW	Sigma-Aldrich
Trimethoprim	SDW and lactic acid	Sigma-Aldrich

2.5 Media

MacConkey agar, Nutrient agar (NA) and broth (NB), Iso-sensitest agar (IST) and broth were obtained from Oxoid (Basingstoke, UK). Lennox L Broth (LB) was purchased from GibcoBRL Life Technologies Ltd. (Paisley, UK) and Chromocult TBX (tryptone bile X-glucuronide) agar from Merck (Darmstadt, Germany). All media were made up with distilled water according to the manufacturer's instructions and autoclaved at 121°C and 15psi for 15minutes prior to use.

2.6 Chemicals buffers and enzymes

Chemicals were supplied by Sigma-Aldrich (Poole, UK) unless otherwise stated and solutions made up with distilled water. Saline was made with 0.85% (w/v) NaCl. All solutions and buffers were sterilised by autoclaving at 121°C and 15psi for 15 minutes prior to use. Restriction enzymes and PCR reagents were purchased from Promega.

Chapter 3. Antimicrobial Susceptibility Testing

3.1 Introduction

The overall aims of this chapter are three fold: firstly, to identify isolates demonstrating interesting resistance phenotypes for further study (for example, to identify resistance to cephalosporins, which are not used on the animals from which these isolates came, or to detect the emergence of a resistance phenotype). Secondly, to determine the prevalence of resistance amongst *E. coli* populations selected on ampicillin, apramycin or nalidixic acid containing agar, to a wide range of antimicrobial agents, and to compare these profiles to isolates taken from an unselected population. Thirdly, resistance profiles were compared using statistical tests to determine whether resistance to an antibiotic conferred resistance to other antibiotics of the same family or to other unrelated antibiotics, and to determine whether plasmid-mediated resistance genes were commonly associated with each other.

In research laboratories, *in vitro* antimicrobial susceptibility testing is commonly performed by the determination of the minimum inhibitory concentration (MIC) of an antibiotic to the test organism. This is considered the gold standard of sensitivity tests (Andrews, 2001), and for research purposes is usually carried out with the test antibiotic incorporated into agar plates. A complete range of antibiotic plates containing doubling dilutions of an antibiotic are prepared by adding agar to freshly prepared antibiotic stock solutions. Bacterial cultures, grown overnight in an orbital shaker, are diluted in sterile saline and a multipoint inoculator is used to replicate test cultures onto the plates. Inoculated plates are incubated at 37°C for 16-20 hours and the MIC (the lowest concentration of antibiotic required to inhibit all visible growth) of the antibiotic is read (Andrews, 2001).

Although this method gives the maximum information about the sensitivity of an isolate to an antibiotic, it is time consuming and expensive (Wheat, 2001), so a compromise method is often employed whereby one or two concentrations of an

antibiotic are used. Commonly the antibiotic is incorporated into filter paper discs or agar (Hutchison, 1954). This method is usually referred to as the 'breakpoint' technique (Wheat, 2001). Understandably, clinical and routine laboratories testing hundreds of isolates often employ disc sensitivity tests, or agar dilution tests using one breakpoint (Edwards, 2003; Wheat, 2001) to enable an isolate to be classified as sensitive or resistant to a drug. Some researchers also use breakpoint techniques where MIC values would be more informative, even when looking at very low numbers (< 40) of isolates (Son *et al.*, 1997).

In this study 410 isolates were to be tested for sensitivity to 25 different antibiotics. To produce MICs for all isolates against all antibiotics would comprise 10,250 tests. Therefore, a breakpoint method of testing was employed whereby a low and high breakpoint concentration of antibiotic was incorporated into agar plates. The use of two breakpoints facilitated the categorisation of isolates into susceptible (S), intermediately resistant (I) and resistant (R) groups. This method had an advantage over using antibiotic discs as it enabled many isolates (n = 33) to be tested on one plate and enabled concentrations of antibiotics relevant to this study to be used.

There is no global standard methodology for MIC tests (Bronzwaer *et al.*, 2002), and many European countries (Germany, The Netherlands, Sweden, Spain, Britain, France and Norway) have issued national guidelines. Methodology may vary in the testing method used (MIC determination by agar dilution or Etest, or disc diffusion), the source of reagents and media, and the interpretive criteria used (Tenover *et al.*, 2001). In Europe, the National Committee for Clinical Laboratory Standards (NCCLS) guidelines are the most frequently followed (Bronzwaer *et al.*, 2002), however The British Society for Antimicrobial Chemotherapy (BSAC) methodology is followed in many British laboratories because it uses MIC ranges and breakpoints most applicable to British clinical isolates. This study had different aims to those of clinical laboratories. One of the aims of this susceptibility profiling was to detect the emergence of reduced susceptibility in *E. coli* isolated from calf rectal faecal samples, not the presence of clinically relevant levels of resistance with any view to treatment. In this way BSAC methodology was followed but breakpoints specific to

this study were used, as those recommended by the BSAC were not suitable. This has the disadvantage that the results generated cannot be directly compared with other studies using these guidelines. However, it is unlikely that the results would be comparable even with BSAC or NCCLS recommended breakpoints, because the method of selecting out isolates for study would bias any prevalence comparisons with other studies.

Phenotypic susceptibility testing has a number of shortcomings (Andrews, 2001; Bergeron & Ouellette, 1998) and inaccuracies of susceptibility testing in Enterobacteriaceae have been detected for disc diffusion (Block *et al.*, 1998), agar dilution, and automated (e.g. MicroScan and Vitek) testing methodologies (Steward *et al.*, 1999). Variation between repeat tests may arise from the use of different media (Andrews *et al.*, 2002; Koeth *et al.*, 2002) or variation in inoculum density (BSAC, 2004). This may be because of differences between spectophotometers or human error in visual judgement of density (BSAC, 2004). With disc diffusion assays the antibiotic content of filter paper discs has been found to vary depending on the manufacturer (Darville & Lovering, 2003). The detection of variation within repeat tests of established and routinely used methodologies emphasises the need for validation of the breakpoint method used in this study and the quantification and analysis of any variation detected. Therefore, this chapter will firstly assess the levels and sources of variation within the susceptibility testing technique used. This was achieved by completion of the following:

a) Quantification of variation between replicate tests.
b) Reproducibility values for this method.
c) Assessment of the sensitivity of the comparison of susceptibility profiles between populations to variation between replicates.

After an assessment of the variation present when using this methodology, the original aims of the susceptibility profiling will be addressed. These are summarised as follows:

a) To determine the susceptibility profiles of bovine commensal *E. coli*, selected from ampicillin, apramycin or nalidixic acid resistant populations.

b) To determine if any associations exist between resistance phenotypes. Resistance genes are commonly found in gene cassettes (Antunes *et al.*, 2004; Heir *et al.*, 2004; Pezzella *et al.*, 2004). These are formed by the action of integrons, which are capable of poaching genes from the environment and incorporating them into bacterial genetic elements (e.g. a plasmid or transposon) (Collis *et al.*, 1993). It was therefore hypothesised that resistance to an antibiotic may be linked to resistance to another unrelated antibiotic as well as to antibiotics of the same family. Association between resistance to antibiotics of the same family, between unrelated antibiotics (i.e. different mechanisms are used to confer resistance), and finally amongst antibiotics to which resistance is commonly plasmid-mediated were examined.

c) To compare the susceptibility profiles of these populations with an unselected population to establish whether certain resistance profiles are specific to a population, and whether isolates from resistant populations more commonly harbour other resistance genes than do isolates from the unselected population.

d) To identify isolates of interest for further study of the molecular mechanisms conferring the resistance phenotype.

3.2 Materials and Methods

3.2a Bacterial isolates

Four hundred and ten bovine commensal *E. coli* were investigated. The numbers of isolates from the unselected population and from each of the antibiotic resistant populations, selected as described in Chapter 2, are shown in Table 3.1.

Table 3.1 Isolates investigated in this study, and the populations and numbers of calves from which they were obtained.

Population	Number of isolates	Number of calves
Unselected (P)	188	9
Ampicillin resistant (Amp^R)	183	9
Apramycin resistant (Apr^R)	12	6
Nalidixic acid resistant (Nal^R)	27	4

To ensure that each plate contained the correct amount of active antibiotic, three different BSAC recommended standard control strains (*E. coli* NCTC10418, *Pseudomonas aeruginosa* NCTC10662, and *Staphylococcus aureus* NCTC6571) exhibiting different levels of sensitivity to the antibiotics were included on every antibiotic plate. The use of three controls maximised the likelihood of spanning the sensitive, intermediate and resistant categories of the antibiotics under test.

3.2b Susceptibility testing

To categorise isolates as being sensitive (S), resistant (R), or of intermediate level resistance (I) to an antibiotic, a high and low breakpoint were used. The breakpoints were chosen to detect the emergence of resistance and identify isolates of interest for further study. They are listed in Table 3.3.

Molten IsoSensitest (IST) agar, cooled to 55°C was added to freshly prepared antibiotic stock solutions. Bacterial cultures, grown overnight in an orbital shaker (at 37°C and 200 rpm) were diluted five hundred-fold in sterile saline and a multipoint inoculator (Denley, UK) used to replicate test cultures onto the plates giving an inoculum of approximately 10^6 colony forming units (cfu) per spot. Inoculated plates were dried and incubated at 37°C for 16-20h. Colonies growing on both the

low and high breakpoint plates were classified as R, those growing only on the low breakpoint plate, I, and those growing on neither were classified S. All isolates were inoculated onto agar plates containing no antibiotic to ensure that any inhibition of growth on test plates was due to the presence of the antibiotic.

3.2c Statistical analyses

In order to explore repeatability and determine sources of variation within the breakpoint methodology employed, susceptibility profiling of each isolate was determined twice. The reproducibility of the results obtained from the replicates was assessed in two ways. Firstly, the level of agreement between replicates was described using the kappa statistic. This semi-quantitative approach measures pairwise agreement between the two repeat classifications, and is corrected for chance agreement. Table 3.2 categorises the ranges of *kappa* according to Everitt (1989). Commonly researchers use 0.6 as a cut off level (Landis & Koch, 1977) with values of 0.6 or greater indicating acceptable levels of variability.

Table 3.2 Categorisation of kappa values according to Everitt (1989).

Kappa value	Category
>0.81	Almost perfect agreement
0.61 – 0.8	Substantial agreement
0.41 – 0.6	Moderate agreement
0.21 – 0.4	Fair agreement
0 – 0.2	Slight agreement

Kappa values test the null hypothesis that there is no more agreement than might occur by chance, but because kappa is a semi-quantitative statistic that does not quantify the significance of any differences between repeat experiments, Wilcoxson signed ranks tests were calculated in addition to kappa values.

Susceptibility profiles were used to study any associations between antibiotics, for example whether resistance to one antibiotic conferred resistance to other antibiotics with the same mechanism of action (same antibiotic family). In order to do this, it was important to establish whether associations between antibiotics would be sensitive to variation in replicate results. This was examined using Pearson's Chi-squared proportions tests, because the associations between antibiotics were to be analysed by comparing proportions of S, I, or R isolates. Two different methods were used to select what data would be used in any subsequent proportions based tests. Firstly, only those results for which replicates were identical would be included in the analysis (method 1). The second approach (method 2) included all results, however where the replicates differed between S and I or I and R, these isolates were allocated into the S or R group respectively. The relative proportions of S, I or R isolates to a particular antibiotic were compared between the two methods. If the relative proportions did not alter between the two methods, method 2 could be used to analyse any associations, thereby allowing the maximum number of isolates to be included in the test giving it more power.

The hypothesis that resistance to an antibiotic was associated with resistance to other antibiotics within the same family, was studied by comparing the proportions of S, I, and R isolates to both the antibiotics being studied, using the *G*-test of association (Sokal & Rohlf, 1995). The same test was used to determine whether associations existed between plasmid-borne antibiotic resistance mechanisms.

To examine the hypothesis that isolates from resistant populations (i.e. Amp^R, Apr^R or Nal^R isolates) were more likely to harbour other resistance genes, proportions of isolates resistant to a particular antibiotic were compared between the resistant population and the unselected (P) population with Pearson's Chi-squared tests. In all statistical analyses, a *P*-value of < 0.05 was taken to indicate significance.

3.3 Results

3.3.a Reproducibility values

The completion of repeat susceptibility profiles for each isolate demonstrated that whilst both repeat results were partially validated by three NCTC control strains there were often discrepancies between the replicates. The reproducibility of the breakpoints used, as assessed semi-quantitatively by the *kappa* statistic are listed in Table 3.3. According to Everitt's criteria (Everitt, 1989) 18 of 25 antibiotics (highlighted in green) demonstrated substantial agreement between repeat experiments.

The Wilcoxson signed ranks tests are also listed in Table 3.3. A *P*-value greater than 0.05 indicates that the replicate means did not differ significantly. Replicate means that did differ significantly have *P*-values less than 0.05, and these are printed in bold.

Table 3.3 shows that although no replicates were S in one and R in another, a significant level of variation is present amongst replicates for 16 of the 25 antibiotics used.

Table 3.3 Repeat classification of isolates, by antibiotic susceptibility at two breakpoint concentrations. Antibiotics are ordered according to decreasing percentage similarity between repeat classifications. *P* values refer to Wilcoxson signed ranks tests. The numbers of isolates that shifted resistance category between repeat experiments are listed in the final two columns.

Antibiotic	Breakpoints (mg/L)	S-S	S-I	S-R	I-S	I-I	I-R	R-S	R-I	R-R	*P*-value	Kappa	%same	Increased resistance	Decreased resistance
Ciprofloxacin	1 / 4	409	0	0	0	1	0	0	0	0	>0.999	1	100	0	0
Sulphamethoxazole	32 / 128	194	0	0	0	0	0	0	0	216	>0.999	1	100	0	0
Enrofloxacin	1 / 4	408	0	0	1	1	0	0	0	0	0.3185	0.6656	99.8	0	1
Tobramycin	2 / 8	393	0	0	3	0	0	0	0	14	0.0835	0.9	99.3	0	3
Trimethoprim	4 / 16	344	1	0	0	0	3	0	2	60	0.4148	0.9459	98.5	4	2
Nalidixic acid	1 / 8	0	3	0	2	345	1	0	1	58	0.7062	0.9335	98.3	4	3
Chloramphenicol	8 / 32	355	4	0	1	0	3	0	0	47	**0.034**	0.9135	98	7	1
Ceftizoxime	2 / 16	370	6	0	5	27	0	0	0	2	0.7636	0.8268	97.3	6	5
Gentamicin	2 / 8	392	0	0	4	2	0	0	8	4	**0.0005**	0.6144	97.1	0	12
Aztreonam	2 / 8	323	0	0	2	34	1	0	13	37	**0.0005**	0.8897	96.1	1	15
Ampicillin	4 / 32	131	11	0	4	3	0	0	1	260	0.1338	0.919	96.1	11	5
Cefotaxime	2 / 16	386	6	0	16	2	3	0	0	0	**0.0331**	0.1304	94.6	9	16
Ceftazidime	4 / 16	330	16	0	6	50	5	0	1	2	**0.0082**	0.7595	93.2	21	7
Penicillin	0.12 / 32	0	0	0	2	137	13	0	28	230	**0.0095**	0.781	89.5	13	30
Azlocillin	16 / 64	54	33	0	9	100	2	0	0	212	**0.0001**	0.824	89.3	35	9
Cefuroxime	8 / 32	312	16	0	5	35	28	0	1	13	**<0.0001**	0.6584	87.8	44	6
Rifampicin	0.5 / 1	1	0	0	0	7	57	0	3	342	**<0.0001**	0.1746	85.4	57	3
Cephalothin	4 / 16	35	24	0	24	224	4	0	15	84	0.1397	0.6987	83.7	28	39
Cefoxitin	8 / 32	259	53	0	15	40	0	0	0	43	**<0.0001**	0.6315	83.4	53	15
Streptomycin	4 / 32	135	2	0	25	60	18	0	56	114	**<0.0001**	0.6289	75.4	20	81
Tetracycline	1 / 64	18	7	0	78	104	12	0	13	178	**<0.0001**	0.5719	73.2	19	91
Azithromycin	0.5 / 4	1	1	0	0	280	101	0	17	10	**<0.0001**	0.0538	71	102	17
Cephradine	8 / 32	132	93	0	36	65	0	0	1	83	**<0.0001**	0.5031	68.3	93	37
Amoxyclavulanic acid	2 / 8	0	6	0	3	84	13	0	124	180	**<0.0001**	0.3199	64.4	19	127
Apramycin	4 / 32	227	9	0	146	16	0	0	0	12	**<0.0001**	0.1625	62.2	9	146

S, susceptible; I, intermediate level resistance; R, Resistant; S-S, I-I, and R-R, the same classification on both replicates; S-I, S-R, I-S, I-R, R-S, and R-I, different classification between replicate experiments. Yellow shading indicates identical classifications between replicates, and green shading indicates substantial agreement between replicates. Significant *P* values are highlighted in bold.

3.3.b Sensitivity of associations between antibiotics to variation between replicates.

Table 3.3 demonstrates that for some of the antibiotics variation occurred between replicate experiments. On account of this variation, two methods were used to select which data would be used in subsequent statistical tests. Method 1 used only those results for which replicates were identical and method 2 included all results but

grouped any isolates with replicates differing between S and I, or I and R as S or R respectively. The impact of using one method over the other, as assessed by Pearson's χ-squared tests, are shown in Table 3.4

Table 3.4 Significance of variation generated by different grouping methods, assessed by Pearson's χ-squared test.

Antibiotic	χ-squared	*P*-value
Ciprofloxacin	0	1
Sulfamethoxazole	0	1
Enrofloxacin	0	1
Tobramycin	0.0004	0.999
Ceftizoxime	0.0116	0.9942
Chloramphenicol	0.0489	0.9758
Rifampicin	0.0116	0.95
Trimethoprim	0.1025	0.9244
Ampicillin	0.496	0.7804
Cefoxitin	1.7562	0.4156
Aztreonam	1.9158	0.3837
Cefotaxime	2.8321	0.2427
Penicillin	2.9848	0.2248
Ceftazidime	3.3239	0.1898
Apramycin	3.5129	0.1727
Gentamicin	3.8429	0.1464
Nalidixic acid	4.974	0.0832
Streptomycin	6.5281	0.0382
Azlocillin	9.3045	0.0095
Cefuroxime	12.7818	0.0017
Amoxyclavulanic acid	15.8838	0.0004
Cephalothin	15.6181	0.0004
Cephradine	18.5139	0.0001
Azithromycin	83.4351	<0.0001
Tetracycline	45.3986	<0.0001

In the lower part of Table 3.4 are listed eight antibiotics with significant *P*-values. For these antibiotics the selection of data by either method 1 or 2 makes a significant difference to the proportions of S, I and R isolates, and so any further statistical tests

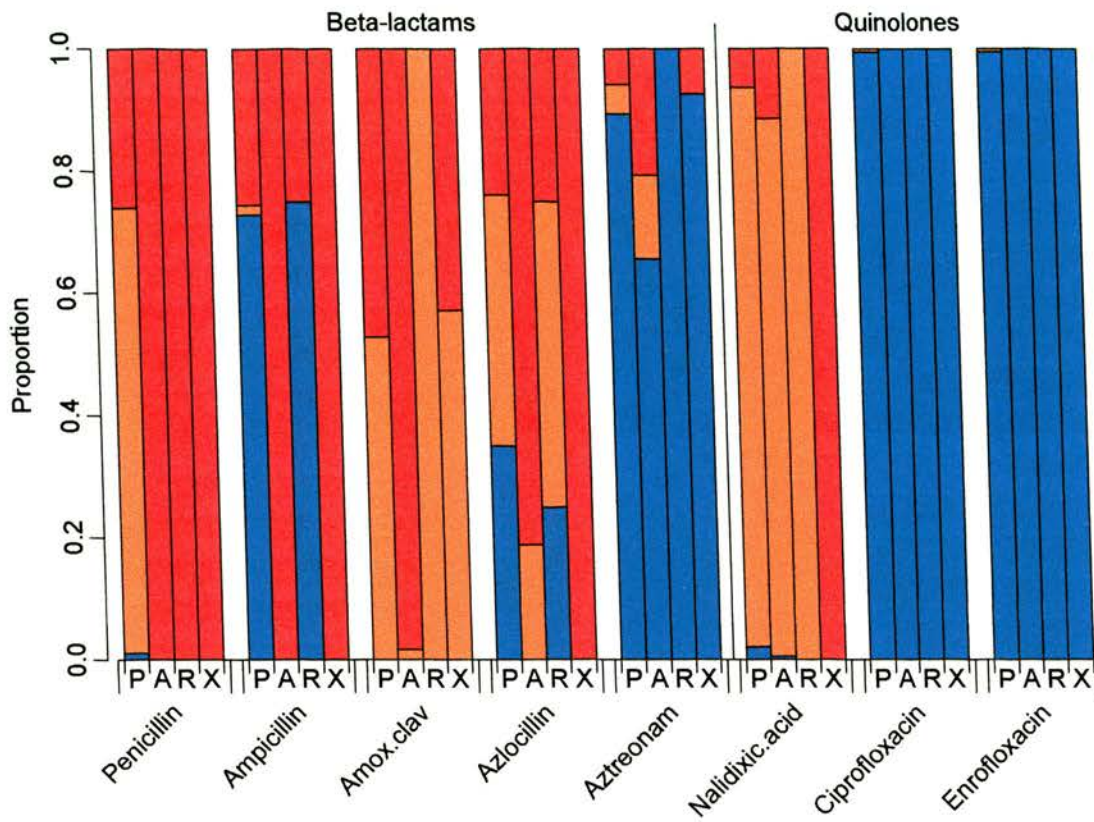
based on relative proportions could only be performed on those data for which repeat experiments yielded the same susceptibility phenotype. Comparing these results with Table 3.3, one can see that all these antibiotics show significant differences between replicate experiments, with the exception of cephalothin. In this case the columns indicating numbers of isolates that shifted resistance category between repeat experiments (columns headed "Increased resistance" and "Decreased resistance") showed similar numbers (28 and 39). A significant difference between replicate classifications is not recorded by the Wilcoxson signed ranks test, because it measures significant differences in a directional manner. In this way, the two shifts cancel each other out, preventing the detection of a significance difference between the replicates.

The proportions of resistant, intermediately resistant and sensitive isolates did not differ significantly between the two different grouping methods ($P > 0.05$) for 17 of the 25 antibiotics tested. For these drugs subsequent statistical analysis based on proportions were performed on data grouped by method 2, permitting the use of the maximum number of results and thereby giving more power to each test.

3.3.c Proportions of resistance

The four populations of *E. coli* (amp^R, apr^R, nal^R, and unselected *E. coli* populations) were analysed to determine the sensitivities of isolates to 25 antibiotics. Figures 3.1 to 3.3 show the proportions of resistance to these antibiotics within the different populations. In these figures P is the unselected population, A, the amp^R, R, the apr^R, and X, the nal^R population. Resistance is shaded red, intermediate level resistance, orange, and sensitive, yellow. Sensitivity is shown as a percentage of the total number of isolates. It is important to note that the number of isolates in the population may affect these proportions. The unselected and amp^R populations are much more comparable than the nal^R and apr^R populations as they comprise similar large numbers of isolates (188 and 183 respectively). The nal^R and apr^R populations only represent 27 and 12 isolates respectively.

Figure 3.1 Comparison of the proportions of resistance of amp^R, apr^R, nal^R and unselected populations to β-lactams and quinolones.



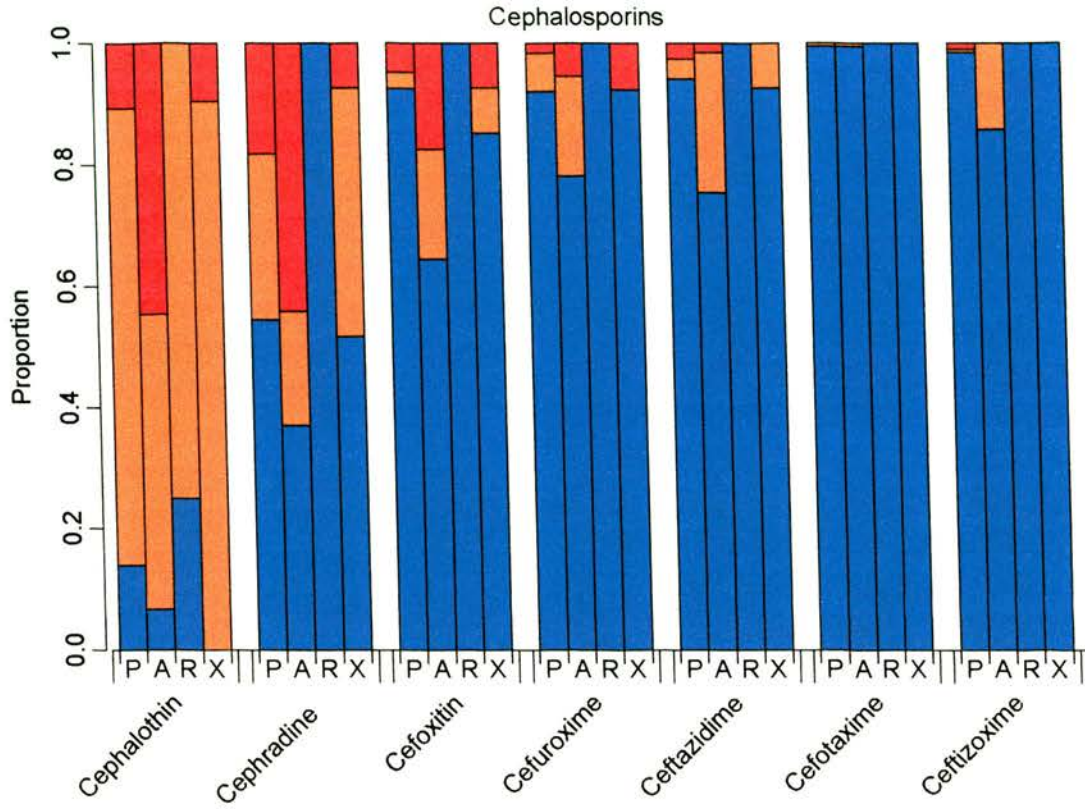
P, unselected; A, amp^R, R, apr^R; X, nal^R; red, resistant; orange, intermediate resistance; blue, sensitive.

Figure 3.1 demonstrates that most isolates (408 / 410) were intermediately or fully resistant to penicillin. The amp^R population had the highest proportion of resistance to amoxyclavulanic acid, azlocillin and aztreonam. The unselected and apr^R populations had similar proportions of resistance to ampicillin (0.26 and 0.25) and azlocillin (0.24 and 0.25). All nal^R isolates (n = 27) were resistant to penicillin, ampicillin and azlocillin, but demonstrated diversity in their susceptibility to amoxyclavulanic acid and aztreonam.

A high prevalence of nalidixic acid resistance (intermediate or high level) was detected in all populations, but only two isolates were intermediately resistant to the fluoroquinolones (ciprofloxacin and enrofloxacin), and these isolates were picked from unselective plates.

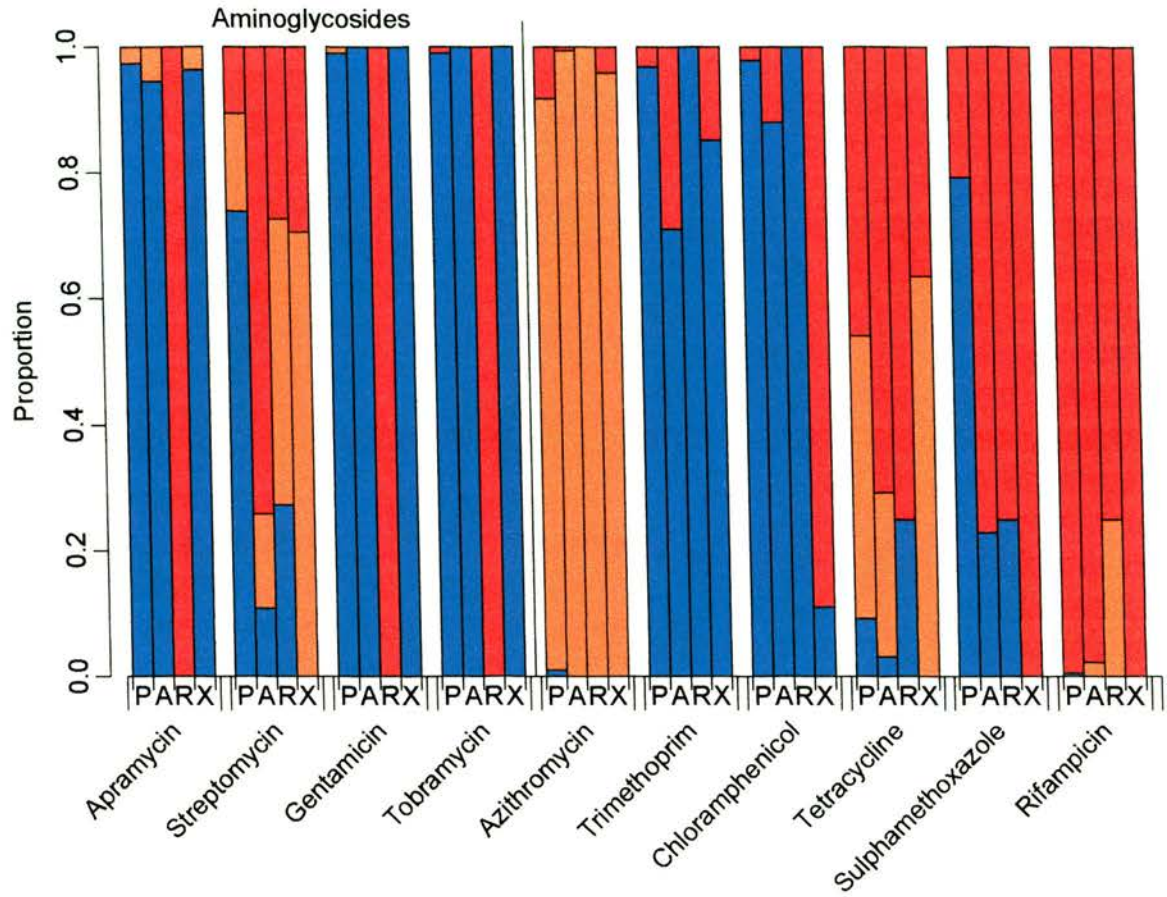
Figure 3.2 Comparison of the prevalence of resistance of amp^R, apr^R, nal^R and unselected populations to the cephalosporins.

P, unselected; A, amp^R, R, apr^R; X, nal^R; red, resistant; orange, intermediate resistance; blue, sensitive.



The graphs in Figure 3.2 are ordered according to the generation of cephalosporin. The 1st generation cephalosporins (cephalothin and cephradine) are on the left hand side, the 2nd generation (cefoxitin and cefuroxime) in the centre, and the 3rd generation cephalosporins (ceftazidime, cefotaxime and ceftizoxime) are to the right. There is a general trend towards increasing susceptibility with the newer (3rd generation) cephalosporins. Resistance (red shading) to ceftazidime and ceftizoxime is more prevalent in the unselected population than the amp^R population.

Figure 3.3 Comparison of the prevalence of resistance of amp^R, apr^R, nal^R and unselected populations to the aminoglycosides and other classes of antibiotic.



P, unselected; A, amp^R, R, apr^R; X, nal^R; red, resistant; orange, intermediate resistance; blue, sensitive.

The apr^R population contained only 12 isolates, however all susceptibility classes were observed when isolates were profiled with streptomycin (Figure 3.3). The vast majority of isolates had decreased susceptibility to rifampicin, because of the barrier function of the gram-negative outer membrane in preventing entry of this antibiotic (Burman *et al.*, 1972).

A greater percentage of amp^R isolates also demonstrate reduced susceptibility to streptomycin, trimethoprim, chloramphenicol, tetracycline and sulphamethoxazole, than did the unselected population. Therefore, it is hypothesised that resistance to ampicillin may be linked to resistance to other antibiotics by the carriage of a multi-drug resistance plasmid. Similarly, the apr^R population has a higher prevalence of tetracycline and sulphamethoxazole resistance than the unselected population, which

may be due to the presence of a plasmid harbouring these resistance determinants within the apr^R population. These hypotheses are examined in the following section.

3.3.d Between-population comparison of susceptibility proportions

This section aims to address the following three hypotheses:

i) Resistance to an antibiotic confers resistance to other antibiotics of the same family.

ii) Isolates from resistant populations more commonly harbour other resistance genes.

iii) Plasmid mediated resistance genes are commonly associated with each other.

i) Resistance to an antibiotic confers resistance to other antibiotics of the same family.

This hypothesis acts as a positive control for the *G*-tests, because resistance to an antibiotic commonly confers resistance to other antibiotics of the same family (Amyes *et al.*, 1996). To examine this hypothesis, the proportions of S, I and R isolates, to one antibiotic were compared to those of another antibiotic from the same antibiotic family (i.e. with the same mechanism of antibacterial action). Representative antibiotics from different families were used in the analysis, the results of which are listed in Table 3.5.

Table 3.5 Association in level of susceptibility between antibiotics of the same family.

Antibiotics compared		*G* value	*P*-value
Amoxyclavulanic acid	Ampicillin	95.76	<0.0001
Amoxyclavulanic acid	Aztreonam	65.49	<0.0001
Amoxyclavulanic acid	Cephalothin	80.79	<0.0001
Amoxyclavulanic acid	Ceftazidime	42.44	<0.0001
Cephalothin	Ceftazidime	169.18	<0.0001
Apramycin	Gentamicin	45.69	<0.0001
Apramycin	Streptomycin	6.35	0.1747

These results support the hypothesis that resistance to an antibiotic confers cross-resistance to other antibiotics of the same family. All tests of association between levels of susceptibility to antibiotics of the same family yielded highly significant positive associations ($P < 0.0001$), except apramycin verses streptomycin. This result was expected because the enzyme conferring resistance to apramycin does not confer cross-resistance to streptomycin (Bräu *et al.*, 1984) even though both antibiotics are aminoglycosides. This test was performed on proportions of S, I and R isolates grouped with either method 1 or method 2, depending on the results of section *c*. As a check, tests were repeated using the more conservative method (method 1), where only those results for which repeat classification yielded the same resistance phenotype. The same significant associations were found.

ii) Isolates from resistant populations more commonly harbour other resistance genes.

Pearson's χ-squared test was used to determine whether isolates from the amp^R and nal^R populations had a significantly greater proportion of resistance to other antibiotics than isolates from the unselected population. The apr^R population was not included in the study as it contained only 12 isolates, which would result in an unreliable test. In this analysis, resistance was classified as either the number of R isolates or the number of non-sensitive (R + I) isolates in the population. Different

definitions of resistance may be used in each comparison as long as the same definition is used within a comparison. This is because the question being addressed is whether there is an association between increases in resistance and the type of population. Representative antibiotics were chosen for this analysis based on their different mechanisms of action, and the results of this analysis are shown in Table 3.6.

Table 3.6 Association between resistance to an antibiotic, and resistant population compared to the unselected population.

		R isolates only		R + I isolates	
Population	Antibiotic	χ^2	*P*-value	χ^2	*P*-value
Amp^R	Apramycin	NA	NA	1.11	0.292
	Nalidixic acid	2.37	0.124	0.757	0.384*
	Trimethoprim	40.98	<0.0001	40.98	<0.0001
	Chloramphenicol	12.45	0.0004	21.919	<0.0001
	Tetracycline	15.58	0.0001	3.285	0.07
	Sulphamethoxazole	115.45	<0.0001	115.45	<0.0001
Nal^R	Ampicillin	54.41	<0.0001	51.17	<0.0001
	Apramycin	NA	NA	0.08	0.777*
	Trimethoprim	4.724	0.03*	4.724	0.03*
	Chloramphenicol	149.33	<0.0001*	149.33	<0.0001
	Tetracycline	0.0451	0.8318	1.4965	0.2212*
	Sulphamethoxazole	66.03	<0.0001	66.03	<0.0001

* Indicates expected frequencies of <5. NA indicates no R isolates were present in one of the populations. R, resistant isolates; I, intermediately resistant isolates.

The ability of the test to detect an increase or decrease in the proportion of resistance between the two populations requires adequate numbers of isolates to be present in the contingency table. This ultimately depends on the ability of the breakpoints used to partition the population, which will differ between antibiotics. The only instance for which the definition of resistance does make a difference to the significance of any association is the presence of tetracycline resistance in the amp^R population. This is because of a large number of isolates in the comparison with intermediate level resistance (41/183 isolates in the amp^R population were intermediately resistant to tetracycline, results not shown). The addition of all I isolates into the R group

dramatically affects the numbers of R isolates used in the test. The results of Table 3.6 indicate that high level tetracycline resistance (MIC ≥ 64mg/L) is significantly associated with ampicillin resistance. Ampicillin resistant isolates were also significantly associated with resistance to trimethoprim, chloramphenicol, and sulphamethoxozole (Table 3.6)

Nalidixic acid resistant isolates were significantly associated with sulphamethoxozole and ampicillin resistance. The significance of the association between nalidixic acid resistance and chloramphenicol and trimethoprim resistance should be interpreted with caution because the expected frequencies were fewer than five.

iii) Plasmid mediated resistance genes are commonly associated with each other.

To examine this hypothesis, the proportions of S, I and R isolates, to either ampicillin or apramycin were compared to those of other antibiotics to which resistance is commonly plasmid mediated. These results are shown in Table 3.7.

Table 3.7 Association in level of susceptibility between antibiotics to which resistance is commonly carried on plasmids.

Antibiotics compared		*G* value	*P*-value
Ampicillin	Apramycin	0.882	0.9271
Ampicillin	Chloramphenicol	38.96	<0.0001
Ampicillin	Sulphamethoxozole	175.45	<0.0001
Ampicillin	Tetracycline	49.83	<0.0001
Ampicillin	Trimethoprim	49.81	<0.0001
Apramycin	Chloramphenicol	3.532	0.171
Apramycin	Sulphamethoxozole	2.848	0.2408
Apramycin	Tetracycline	13.36	0.0096
Apramycin	Trimethoprim	3.966	0.1377

Resistance to ampicillin is significantly associated with resistance to chloramphenicol, sulphamethoxazole, tetracycline and trimethoprim (Table 3.7). These conclusions support those of section ii (Table 3.6). Apr^R isolates were only significantly associated with tetracycline resistance indicating the presence of an apramycin resistance plasmid that also harbours tetracycline resistance. All associations were positive.

3.4 Discussion

This study aimed to determine the susceptibility profiles of 410 isolates to 25 different antibiotics. The number of tests involved meant that the determination of full MICs for each isolate was not practical, and so a breakpoint method of testing was used. By repeating each profile twice, variation arising from this methodology could be measured.

3.4a Assessment of levels and sources of variation within the methodology

Although three control organisms recommended by the BSAC (Andrews, 2001) were included in each experiment (NCTC 10418, NCTC 6571 and NCTC 10662) a significant level of variation was detected with 16 antibiotics, and a less than satisfactory level of reproducibility was observed with seven. Some experimental errors may have remained undetected if the MICs of these standard strains were not close to the breakpoint concentrations chosen.

Discrepancies between replicates can arise from slight variations in inoculum, degradation of antibiotic over time, and variation in reading the plates (BSAC, 2004), but this should be minimal if a single protocol is adhered to and results are recorded by the same operator, as was done in this study. The variation observed is due to the positioning of the breakpoints. The effect of breakpoint positioning on classification is illustrated in Figure 3.4.

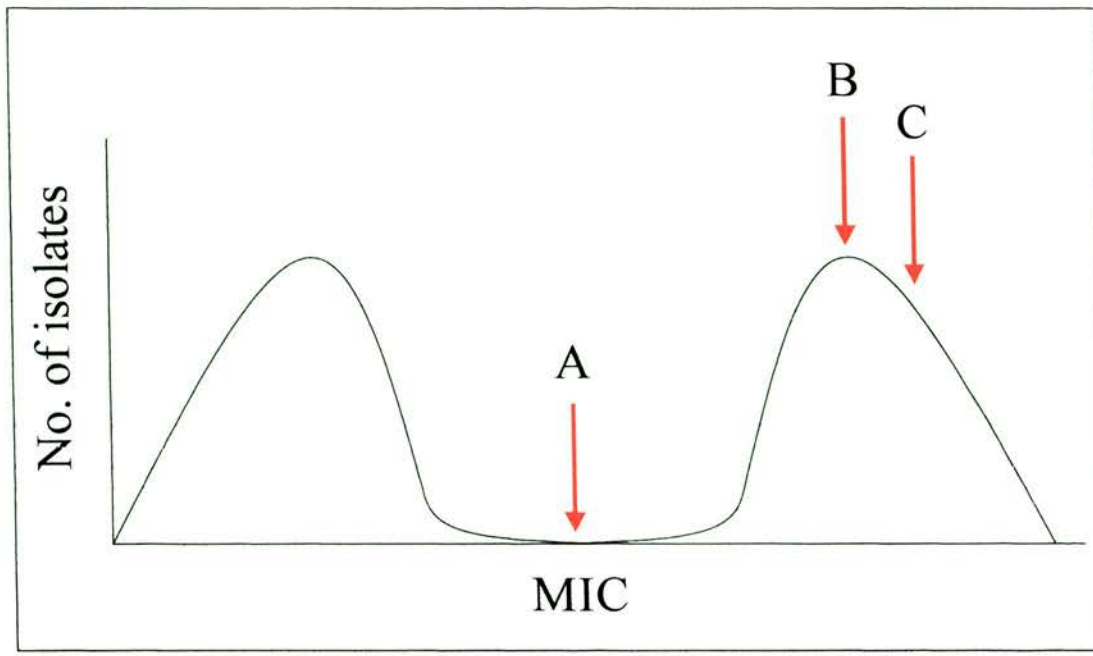


Figure 3.4 Variation in susceptibility classification due to breakpoint position.

Figure 3.4 shows a population of bacteria with a range of susceptibilities to an antibiotic, given as MIC values. The population comprises two discrete groups, those that are sensitive, and those that are resistant to the antibiotic. This bimodal distribution is typical of a number of antibiotics used in this study, and is caused by the acquisition of a resistance mechanism resulting in a characteristic increase in MIC. If a breakpoint is positioned at point A, any sight variations in concentration of antibiotic because of degradation of the drug or inaccuracies in making the antibiotic plates will not affect the number of isolates classified as R or S. In contrast, if the breakpoint is positioned at point B, slight variations in antibiotic concentration may result in concentration C being the actual concentration of antibiotic used. This will result in a decrease in the number of isolates that are classed as R, and a corresponding increase in those classed as I. With prior knowledge as to the range of MICs expected within a given population, breakpoint concentrations can be used which, like concentration A, split the population into discrete groups. No information was available on the susceptibilities expected from the isolates examined in this study. To acquire this knowledge would require the completion of full MICs on a very large subset, if not all of the isolates. This is because the isolates are expected to

have extremely varied susceptibility profiles because some were selected on antibiotics prior to isolation and others were not.

The presence of variation between replicates results from the position of the breakpoints used. The extent of the variation observed also differs between the different antibiotics. This is because it depends on the MIC of the majority of the isolates to that antibiotic and the mechanism of resistance to the antibiotic.

According to the BSAC (Andrews, 2001) a variation of plus or minus one two-fold dilution is acceptable when assessing whether a control strain in a sensitivity test has its correct MIC. In this study, if the majority of isolates had an MIC to a particular antibiotic that was at the breakpoint concentration used, repeat experiments could easily result in two different resistance phenotypes being reported because of minor experimental errors. This may explain why many isolates were sensitive to apramycin, azlocillin, cefoxitin, cephradine, or tetracycline in one experiment and demonstrated intermediate level resistance in the repeat experiment.

The isolates in this study varied in their susceptibility to different classes of antibiotic, because of the initial selection of isolates either on media containing an antibiotic or on media without antibiotic supplementation. This range of susceptibilities is a good way of testing the true ability of the breakpoints used to classify isolates into resistance phenotypes. Quality control of susceptibility testing has in the past been conducted with a high proportion of susceptible isolates (Chen *et al.*, 1995) resulting in an unfair test of the methodology.

The mechanism by which bacteria acquire resistance to an antibiotic is also of importance when assessing the effect of variation due to breakpoint position. The categorisation of isolates into the three groups with very few discrepancies between repeats will be obtained where the presence of a resistance mechanism results in large increases in MIC, forming discrete populations, and the breakpoints used have been at positions suitable for distinguishing these populations.

Sulphonamide resistance is mediated by the production of dihydropteroate synthetase with reduced affinity for sulphonamides, but not para-aminobenzoic acid (a precursor in the bacterial metabolic pathway for the synthesis of tetrahydrofolate) (Wise & Abou-Donia, 1975). In this way, the inhibitory effect of the antibiotic is bypassed resulting in a dramatic increase in MIC. The difference in MIC between sensitive and resistant isolates is large enough that no I isolates were identified with these breakpoints. Therefore, the breakpoints used must be both present in the trough of the bimodal curve resulting in unambiguous categorisation. This may also be the case with trimethoprim, where there are few (n = 6) discrepancies between replicates. Trimethoprim resistance occurs by the production of an altered dihydrofolate reductase enzyme (the trimethoprim target) (Amyes & Smith, 1974) resulting in a large increase in MIC.

In contrast, Livermore & Chen (1999) describe the occurrence of a high frequency of resistant *P. aeruginosa* isolates in UK laboratories being reported as sensitive. The high rate of miscategorisation was thought to be due to the unimodal distribution of MIC values. Where a breakpoint cuts the tail of a unimodal distribution, resistance classification by this breakpoint is prone to be erratic, compared to a breakpoint present in between the two discrete populations of a bimodal distribution. This is likely to be the reason for discrepancies in categorisation of susceptibility, and hence the low Kappa values, of the breakpoint tests used in this study to apramycin, cephradine and amoxyclavulanic acid. Here different resistance mechanisms can occur resulting in different MIC values. These include enzymatic modification of apramycin, enzymatic degradation and reduced permeability of the β-lactams and cephalosporins (Moosden, 1997), and efflux pumps capable of removing these antibiotics (Edgar & Bibi, 1997; Rosenberg *et al.*, 2000). This will lead to a greater number of possible MIC values present in the population. Efflux pumps may also play a role in resistance to the quinolones, chloramphenicol and other β-lactams (Sulavik *et al.*, 2001), but the presence of variation between replicates again depends on the position of the breakpoints in relation to the range of MIC values in the population.

The extent of the variation occurring between replicate experiments differed between the antibiotics. This needed to be considered before examining the presence of any associations between resistance phenotypes. Recently, statistics have been applied to the determination of associations between cyclohexane resistance and multiple antibiotic resistance (Randall *et al.*, 2001). Here isolates were classified as S or R based on the results of disc diffusion assays, but the presence of any variation in the categorisation of susceptibility was not examined prior to the determination of any associations between resistance phenotypes. The identification of antibiotics for which the classification was ambiguous would have been a useful addition to the study.

3.4b Proportions of resistance

In the unselected population, resistance amongst the ß-lactam antibiotics was detected against penicillin, ampicillin, azlocillin and amoxyclavulanic acid, which indicates that these strains represent a substantial proportion of the calves' intestinal flora. The sampled calves were not treated with any ß-lactams, but penicillin was used to treat two calves reared with the sampled calves. The detection of β-lactam resistance in a high proportion of commensal *E. coli* may reflect cross-contamination of resistant strains between treated and untreated calves, or a high prevalence of β-lactam resistant strains in the calves' immediate environment. Environmental sampling and resistance phenotyping would be required to test this hypothesis.

As was expected, many of the isolates selected on ampicillin plates showed cross resistance to other ß-lactams, although 120 / 183 of this population were sensitive to aztreonam suggesting that the predominate ß-lactamases do not hydrolyse this monobactam.

A low proportion of isolates were resistant to the 3rd generation cephalosporins (eight isolates were ceftazidime resistant and two were ceftizoxime resistant). Surprisingly, resistance to these antibiotics was more common in the unselected population than

the amp^R. Resistance to the 3rd generation cephalosporins commonly occurs by mutation of the β-lactamase genes *bla*$_{TEM-1}$ or *bla*$_{SHV-1}$ causing an extended spectrum of activity of the corresponding enzyme (Bradford, 2001). Therefore, TEM-1 and SHV-1 enzymes were expected to be more prevalent in the amp^R population than those from the unselected population. The mechanism of resistance may be the presence of a cephaloporinase (Alvarez *et al*, 2004; Zhao *et al*., 2001) or an efflux pump (Nikaido *et al*., 1998). This is discussed in more detail in Chapter 8.

Only two isolates demonstrated reduced susceptibility to the fluoroquinolones enrofloxacin and ciprofloxacin. These were picked from unselective plates. Resistance to the quinolones in *E. coli* commonly occurs by mutation in the *gyr*A gene (Oram & Fisher, 1991), or reduced levels of quinolone accumulation (Cohen *et al*., 1988; Hirai *et al*., 1986a). A single mutation in the quinolone resistance determining region of *gyrA* confers high level nalidixic acid resistance and low level ciprofloxacin resistance (MIC ≤ 4mg/L) (Vila *et al*., 1994). This would explain the resistance phenotype observed, but it is surprising that none of the isolates from the nal^R population demonstrated this phenotype. Alternatively, the intermediate level resistance to the fluoroquinolones in these two isolates may be due to a reduction in quionolone accumulation. A decrease in the number of OmpF porins (the outer membrane protein channel through which fluoroquinolones are thought to diffuse) has previously been correlated with a 4-fold increase in resistance to both nalidixic acid and ciprofloxacin (Hirai *et al*., 1986a; Hirai *et al*., 1986b). Active efflux of quinolones has also been found to contribute to the final MIC of a strain to varying degrees (Everett *et al*., 1996; Martinez *et al*., 1998). Sequencing of the QRDR of the *gyrA* gene, studies of drug accumulation, and outer membrane protein western blots would be required to test these hypotheses.

Although the apr^R population comprised only 12 isolates, all levels of streptomycin resistance (S, I, and R) were observed. This indicates variation in the mechanism of streptomycin resistance within the apr^R isolates. A number of different streptomycin resistance genes have been identified (Heinzel *et al*., 1988; Hollingshead & Vapnek, 1985; Scholz *et al*., 1989) in addition to resistance due to ribosomal alteration (Traub

& Nomura, 1968) and loss of permeability (Zelazna-Kowalska, 1977), all of which may contribute to the variations in resistance profiles observed within this small group.

3.4c Associations between phenotypes

The prevalence graphs comparing resistance phenotypes between the four populations, indicated that the nal^R population harboured resistance to ampicillin, sulphamethoxozole, chloramphenicol and trimethoprim, more commonly than the unselected population. When this was examined statistically a significant positive association was found between these four antibiotics and the nal^R population ($P < 0.05$). This association is unlikely to be due to any specific propensity for strains harbouring a chromosomal mutation conferring nalidixic acid resistance to acquire these resistance genes. These results can be explained by the presence of a limited number of genotypes in the nal^R population, of which the most prevalent strain also harbours multiple antibiotic resistances. If few other nal^R genotypes are present the results of the test will be biased. The extent of clonality within the nal^R population is examined by PFGE in chapter 4.

The prevalence graphs also indicated that resistance to trimethoprim, chloramphenicol, tetracycline, and sulphamethoxazole is more common in amp^R isolates than isolates from the unselected population. Again, when these potential associations were examined statistically, a positive association was found between each of these antibiotics and the amp^R population ($P < 0.05$). This is likely to be due to the presence of (a) multidrug resistance plasmid(s), which are commonly found in isolates from food-producing animals (Marshall *et al.*, 1990; Mee & Nikoletti, 1983; Oppegaard *et al.*, 2001). The highly significant level of association between these resistance genes and ampicillin resistance, detected by the *G*-test of association also supports this hypothesis.

The ability of integrons, present in mobile elements, to acquire antibiotic resistance genes from other replicons is well documented (Bass *et al.*, 1999; Collis *et al.*, 1993). This results in the formation of gene cassettes (reviewed by Recchia & Hall, 1995) containing a number of resistance genes, either on conjugative transposons, or transposons within plasmids (Antunes *et al.*, 2004; Heir *et al.*, 2004; Pezzella *et al.*, 2004). To examine whether or not the antibiotic resistances identified are present on one plasmid, transfer frequencies can be compared when the different antibiotics are used as selective agents for transfer. This method is described in more detail in chapter 5, where transfer frequencies were used to determine which resistance genes were present on apr^R plasmids.

The apr^R population had a higher prevalence of tetracycline and sulphamethoxazole resistance than the unselected population, which may be due to the presence of a plasmid harbouring these resistance determinants within the apr^R population. When the significance of any associations between apramycin resistance and other plasmid mediated resistance genes were assessed using the *G*-test, a significant association was only found between apramycin and tetracycline resistance. This suggests that within the apr^R population is a plasmid carrying apramycin and tetracycline resistance. Because no apramycin (or other aminoglycosides) or tetracycline was used to treat the animals from which these isolates came, the presence of this plasmid is of interest, and is the focus of chapter 5.

3.5 Concluding remarks

The use of two breakpoint concentrations of an antibiotic allowed a large number of isolates (410) to be screened for susceptibility where the usual practice of determining actual MICs by doubling dilution would be unmanageable. Although different degrees of variation were present within replicate experiments, quantification of this variation permitted valid conclusions to be drawn, and enabled the identification of a number of isolates of interest for further study.

These included the detection of intermediate or high level resistance to 3rd generation cephalosporins. These isolates may harbour ESBLs, previously undetected in Scottish food animals, and will be examined in molecular detail in Chapter 8.

The limited diversity of resistance phenotypes within the nal^R populations suggested a greater degree of clonality within these isolates compared to the amp^R, apr^R and unselected populations. The extent of this possible clonality is examined in Chapter 4 using a combination of genotyping by pulsed-field gel electrophoresis, and mathematical estimates of population diversity.

Positive associations between a number of resistance determinants were identified, suggesting the existence of multidrug resistance plasmids. The presence of a plasmid harbouring apramycin and tetracycline is of special interest, as there appears to be no obvious selective pressure maintaining these plasmids. A detailed study on the types of apramycin resistance plasmid, their spread, and the competitive fitness cost of a bacterium harbouring an apr^R plasmid is described in Chapters 5 and 7.

Chapter 4. Genotyping

4.1 Introduction

The epidemiology of antibiotic resistance in pathogenic *E. coli* is well documented (Chaslus-Dancla *et al.*, 1991; Hartman *et al.*, 2003; Oethinger *et al.*, 1998; Kern *et al.*, 2002; Shen *et al.*, 1999), but much less is known about the epidemiology of antibiotic resistance in commensal *E. coli*. To study this requires a prior understanding of the epidemiology of the commensal *E. coli* at the sub-species level. This includes the estimation of strain diversity within this population, and information on factors that may affect diversity (for example, calf age, antibiotic treatment and diet). This chapter aims to achieve a better understanding of the diversity of *E. coli* present within the calf study group. The genotyping results presented in this chapter will also be used in chapter 5 to aid the understanding of the epidemiology of apramycin resistance.

This introduction comprises two parts. Firstly, the different methods of typing bacteria will be discussed, with justification for the method chosen in this study. Secondly, the specific aims of this chapter will be described in detail.

The species *E. coli* is commonly described as being clonal (Ochman & Selander, 1984; Ørskov *et al.*, 1990; Selander & Levin, 1980). A clone is defined as "any microbial isolate belonging to a set of microbial isolates that have been recovered independently from different sources, in different locations, and perhaps at different times, but showing so many identical phenotypic and genetic traits that the most likely explanation for this identity is a common origin" (Eisenstein, 1990).

As more advanced typing techniques become available, a greater degree of diversity is being detected within this species, bringing into question this idea of clonality within the *E. coli* species. Clonality between strains can never be absolute as it depends on the discriminatory power of the techniques used to type the isolates. The more discriminatory the technique, the more certain one will be of any observed

clonality. Therefore to examine strain diversity within the *E. coli* population, and look for clonal relationships, a highly discriminatory method of distinguishing strains is required.

Before the advent of genotypic methods, epidemiology of *E. coli* was examined by serotyping and antibiotic resistance profiling (Eisenstein, 1990). These methods may be useful in studies of the pathogenesis of infections or in patient management (Gemmell, 1999), but are inadequate for exploring *E. coli* population structure. Antibiotic resistance markers, if present on transferable plasmids, may be spread horizontally under selective pressure or may be lost in the absence of selection (Jones *et al.*, 1980), and a substantial amount of chromosomal variation has been detected within some serotypes (Whittam *et al.*, 1993). These factors mean that genotypic approaches are essential for studying *E. coli* population diversity.

A number of genotypic typing methods are now in use in both clinical and research laboratories. Ribotyping is based on restriction fragment length polymorphism of ribosomal RNA genes (Tarkka *et al.*, 1994) and has been applied to the differentiation of *E. coli* O157 isolates from other *E. coli* (Hahm *et al.*, 2003). The differentiation of *E. coli* O157 was successful by this method, which also had the advantage of identifying three isolates that were misclassified as *E. coli* by the cultivation method used. However, ribotyping has been reported to be ineffective at discriminating between isolates within the O157:H7 serotype (Martin *et al.*, 1996). The recent use of ribotyping and multilocus enzyme electrophoresis (MLEE) (where strains are typed by the electrophoretic mobility patterns of multiple metabolic enzymes) (Selander *et al.*, 1986), to determine the clonal structure of avian *E. coli* strains, highlighted MLEE as a better method to discriminate between strains (Silveira *et al.*, 2003). The main disadvantage of MLEE is that typing is based on the structure of between 5 and 15 enzymes, rather than the whole genome.

A number of PCR-based methods are also used in bacterial phylogenetics, including random amplified polymorphic DNA (RAPD) (Power, 1996), arbitrary fragment

length polymorphism (AFLP) (Vos *et al.*, 1995), and repetitive element PCR (rep-PCR) (Versalovic *et al.*, 1991).

RAPD-PCR uses short primers with sequences not directed at any specific genomic sequence, which hybridise at random sites in the DNA to initiate amplification (Welsh & McClelland, 1990). This method was applied to the typing of *E. coli* from beef cattle faeces, carcasses and ground beef (Aslam *et al.*, 2002), leading the authors to conclude that *E. coli* contamination of ground beef originated from cattle faeces. However, RAPD-PCR was found to be less reproducible and less discriminatory than PFGE when these two methods were used to trace fluoroquinolone resistant *E. coli* isolated from cancer patients (Tascini *et al.*, 1999). Other criticisms of RAPD-PCR have been described, including variation in fingerprints due to DNA or primer concentration and staining methodology (Van Belkum *et al.*, 1993), and difficulties in comparison of patterns between gels and between laboratories (Power, 1996).

Other PCR based methods, although relatively cheap and rapid, are also associated with problems that limit their use for studying the population diversity of *E. coli*. AFLP-PCR uses PCR primers generated by enzymatic digestion of the DNA, which are then used in subsequent PCR of other isolates (Vos *et al.*, 1995). Problems arise if the DNA used is not highly purified and of high quality, leading to artificial amplification (Mueller & Wolfenbarger, 1999). The production of an insufficient number of fragments may also lead to incorrect conclusions of clonal identity (Mueller & Wolfenbarger, 1999).

Rep-PCR is based on the amplification of repetitive intergenic consensus sequences, and was used to type pathogenic and non-pathogenic *E. coli* (Johnson & O'Bryan, 2000). In this study rep-PCR failed to resolve all the major pylogenetic groups previously identified by MLEE, significantly limiting the use of this methodology as a typing tool for *E. coli*. Although here one may argue that the use of a genotypic typing method (i.e. rep-PCR) is a more accurate way to determine genetic relatedness than MLEE, as the latter does not compare the genome directly, but compares the structure of 5 -15 proteins expressed by the genome (Dijkshoorn & Towner, 2001).

In a comparative study, AFLP, rep-PCR, ribotyping and PFGE were applied to the subtyping of foodbourne and environmental *E. coli* isolates (Hahm *et al.*, 2003). All methods were able to differentiate the O157 serotype, but the phylogenetic relationships between the isolates differed depending on the typing method chosen. In this study, PFGE was found to be the most effective method of subtyping *E. coli* O157. Noller *et al.* (2003) also report similar findings recommending the use of PFGE typing.

Taking into account the advantages and limitations of the typing methods used previously to study *E. coli* population structure, PFGE was chosen as the method with which to type the commensal *E. coli* in this work. The perceived problems associated with this technique are that it is relatively expensive and labour intensive, and where highly diverse patterns are found it cannot be used to determine evolutionary relationships. There are few studies where this method has been used to type commensal *E. coli*, but it is a highly discriminatory method and the results are extremely reproducible (Osek 2000).

This chapter aims to address four hypotheses, which are outlined below.

1. Commonly, one isolate per faecal sample is examined in epidemiological studies, however it is hypothesised that different genotypes are present in one faecal sample. How commonly this occurs will give an indication of whether or not one isolate per faecal sample is adequate for epidemiological studies.
2. Population diversity can be quantified by statistical estimates. Ecologists and evolutionary biologists have developed statistical approaches to estimating diversity within and between populations (Chao, 1987; Colwell & Coddington, 1994; Martin 2002). To test whether these methods are applicable to the microbial diversity data in this chapter, both Simpson's diversity index, and the Chao1 richness estimator will be used. Once the different methods for quantifying diversity have been explored, they will be used, in addition to non-quantitative graphical methods, to test hypotheses 3.

3. Bacteria may become resistant to ampicillin and apramycin by the acquisition of a plasmid (Chaslus-Dancla *et al.*, 1991; Heffron *et al.*, 1975). In contrast, nalidixic acid resistance is commonly acquired through mutation of the gyrase and topoisomerase genes (Vila *et al.*, 1994). As the mechanisms of resistance acquisition between the resistant populations are different, it is hypothesised that population diversity will also differ between these populations. The unselected population is hypothesised to be the most diverse because it comprises all the other resistant populations in addition to any non-resistant strains.
4. Lastly, because the total numbers of *E. coli* have previously been found to decrease with increasing calf age (Hoyle *et al.*, 2004) it was hypothesised that the population diversity may also change as the calves mature. The intestine of a newborn calf is initially sterile, but is rapidly colonised by different strains from both the environment and from other calves and dams (Hartyl & Dykhuizen, 1984). In ecological terms, Andrews & Hall (1986) consider a given population to comprise two different strategists, those that rely on high rates of reproduction to survive in the community (*r* strategists), and those that rely on optimal utilisation of available resources (*K* strategists). Selection for *r* strategists occurs in un-crowded conditions, but when resources are limited their populations crash. In contrast, *K* strategists depend on specific adaptations to an environment and therefore compete better in crowded and resource limited conditions. The *E. coli* population diversity is hypothesised to decrease with calf age because the early colonisers, most of which will be *r* strategists, are eventually out competed by *K* strategists, which have specific adaptations to the environment and tend to be more stable and permanent members of the community (Atlas & Bartha, 1998). This hypothesis will be tested statistically to determine whether the numbers of genotypes detected change significantly with increasing calf age.

4.2 Materials and Methods

4.2a Bacterial isolates

The isolates used in this study were collected as described in chapter 2. Table 4.1 lists the isolates used in this study, sampling dates, and the calves from which they were obtained. In total 543 commensal *E. coli* were examined by PFGE, comprising 139 unselected isolates, 208 amp^R, 45 apr^R and 151 nal^R isolates.

Table 4.1 Numbers of isolates from each population and calf, typed by PFGE.

Calf	Sampling date	Number of isolates genotyped			
		unselected	amp^R	apr^R	nal^R
684	14.09				1
	17.09	6	8		12
685	17.09	2	2		2
687	17.09	24			
	26.09	9	4		1
	1.10	6	5		
	9.10	6	4		
	17.10	4	4	10	1
	24.10			10	
	12.11			3	
689	17.09	4	6		6
	26.09				4
	1.10				1
	19.11		1		
690	18.09	10	23		
	26.09	2	12		
	1.10	5	9	2	12
	9.10	6	20		5
	17.10	4	3		12
	24.10		9		4
	29.10		4		
	12.11			1	
	19.11		1		
691	18.09		17		
693	26.09	6	9	4	7
	1.10	6	5		5
	9.10	6	6		15
	17.10	9	6		
694	26.09	6	6	8	6
	1.10	5	4		
	9.10	6	12		16
	17.10	7			
696	1.10				4
	9.10				2
	17.10		1		4
	24.10				8
	12.11			6	2
	19.11		1		
698	1.10				6
	24.10		14		
	29.10		12		
	10.12			1	
699	1.10				8
	9.10				5
	29.10				2

4.2b Pulsed-field gel electrophoresis (PFGE)

PFGE was performed as described by Gautom (1997). Briefly, a single colony or cryopreservative bead was used to inoculate 10 ml LB broth for incubation at 37°C with shaking (150 rpm) for 16 to 18 hours. Cultures were harvested by centrifugation at room temperature at 2,190 x *g* (3,600 rpm) for 12 min. The cells were washed in SE (75mM NaCl, pH 8.0; 25mM EDTA, pH 8.0) by centrifugation and the optical density adjusted to 1.40 at a wavelength of 610nm. Chromosomal-grade agarose (Bio-Rad Laboratories, Hercules, Calif.) was prepared in TE buffer (10mM Tris; 1mM EDTA, pH 8.0) to a final concentration of 1.5%. Equal volumes of agarose and cell suspension were mixed and dispensed into plug moulds (Bio-Rad Laboratories). Following solidification, plugs were transferred into lysis buffer (50mM Tris, pH 8.0; 50mM EDTA, pH 8.0; 1% sarcosine; 1 mg proteinase K per ml) for 24 h incubation at 55°C. Plugs were then washed eight times with TE for 15 min at 4°C, and then left overnight in TE at 4°C. Unless used that day, plugs were stored in TE at 4°C for up to three months. One third of a plug was then preincubated in 200µl of 1 x *Xba*I buffer (Promega) for 1 hour at room temperature. The buffer was removed and replaced with a fresh mixture containing 50U of *Xba*I (Promega) in 1 x restriction buffer and incubated at 37°C for 16 to 20 hours. Following a brief wash in 0.5M Tris-borate-EDTA (TBE) (45mM Tris base, 45mM boric acid, 1mM EDTA, pH 8.0), electrophoresis of the samples was performed on the CHEF-mapper (Bio-Rad Laboratories) system using pulsed-field certified agarose (Bio-Rad Laboratories) with 2 litres of 0.5M TBE running buffer (45mM Tris base, 45mM boric acid, 1mM EDTA, pH 8.0). A lambda ladder PFGE marker (New England Biolabs) was run between every six samples. The following electrophoretic conditions were used: initial switch time, 2.1s; final switch time, 54.2s; run time, 22 hours; angle, 120°; gradient, 6.0 V/cm; temperature, 14°C; ramping factor, linear. After electrophoresis the gels were stained for 30 min in 300ml of sterile distilled water containing 300µl of ethidium bromide (1mg/ml), destained for 1 hour in 1 litre of sterile distilled water, and then photographed with the Gel-Doc 2000 system (Bio-Rad).

4.2c Cluster analysis

TIF images of the PFGE gels were exported into BioNumerics software for further analysis and comparison. PFGE fingerprints were compared using the Dice similarity coefficient and clustered by the unweighted pair group method using arithmetic averages (UPGME). PFGE patterns were interpreted according to criterion suggested by Tenover *et al.*, (1995).

Optimisation and position tolerances, calculated with BioNumerics software, were set at 1%. 629 PFGE patterns (86 of which were repeats to check consistency) from the commensal *E. coli* isolated in this study, in addition to 150 copies of the lambda ladder PFGE marker were used in the determination of the optimal position tolerance and optimisation settings. Similarity matrices were calculated for all entries in the database with a range of position tolerance values. The optimal value is that which resulted in high similarity scores within groups of the same genotype (or repeat lanes of the lambda ladder PFGE marker), and as low as possible similarity scores between groups of different genotypes. The optimal values were also able to distinguish similar types within a genotype, i.e. subtypes of a genotype, for example, with one band difference. The optimisation settings were calculated in the same way. With the 543 isolates used in this study, a position tolerance and optimisation value of 1% permitted easy and clear identification of different genotypes, but also gave good specificity within a genotype, as isolates with one band difference were clustered within a genotype.

4.2d Statistics

Genotype richness was estimated using the Chao1 richness estimate. This estimator has been found to be useful for microbial data sets (Hughes *et al.*, 2001), which are commonly skewed towards the low abundance classes. Genotype richness was estimated according to the equation;

$$S_{Chao1} = S_{obs} + \frac{n_1^2}{2n_2}$$

where S_{obs} is the number of species or genotypes observed, n_1, the number of genotypes observed once, and n_2, the number of genotypes observed twice.

Population diversity was quantified by Simpson's diversity index;

$$C = \sum_{i=1}^{s} \frac{n_i(n_i - 1)}{N(N-1)}$$

where n_i is the number of individuals of the ith species or genotype, and N is the number of individuals or genotypes in the population. In this chapter, $D = 1\text{-}C$ is given as the index of diversity because it increases with increasing diversity. This index measures the probability that two isolates taken at random from the population will be of the same genotype.

The effect of calf age, sampling date, and population on genotype richness was analysed with generalised linear models (Crawley, 2002). Population was split into two categories; selected (i.e. antibiotic resistant) or unselected.

4.3 Results

4.3a Population diversity and richness

Population diversity may be expressed as the number of genotypes present (genotype richness), or as the evenness of spread of the genotypes present (diversity). In this chapter the terms richness and diversity will be used to distinguish these two meanings. The genotype richness within the unselected, amp^R, apr^R and nal^R populations was studied using all typed isolates irrespective of calf or sampling date.

Figures 4.1a-d show dendrograms of the different genotypes present within each population. Subtypes within six of the genotypes were detected (with ≤ 2 band differences from the majority type of a genotype), and these are also included in the figures. The subtypes within a genotype will be described and discussed in section 4.3b; genotypic diversity.

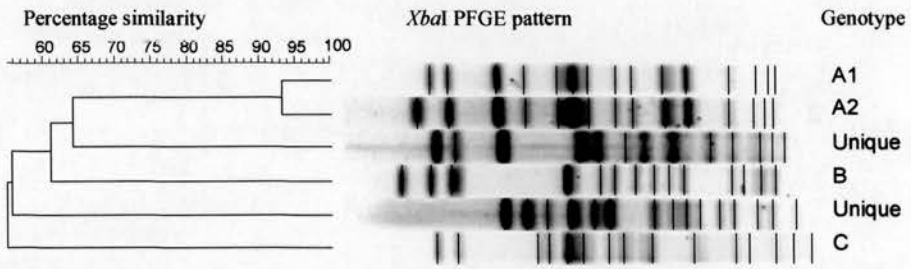


Figure 4.1a PFGE genotypes in the apramycin resistant population. Percentage similarity is given on the scale above the dendrogram. Subtypes of a genotype are designated a number after the genotype letter.

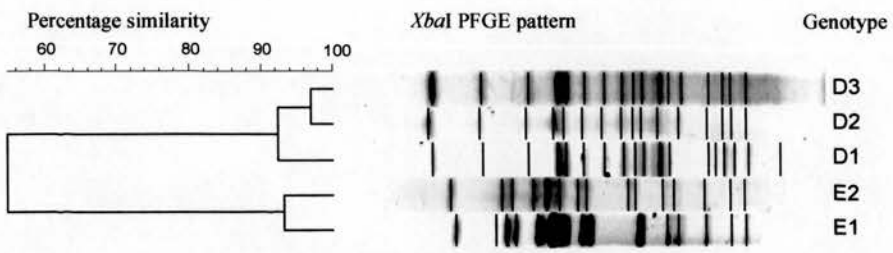


Figure 4.1b PFGE types in the nalidixic acid resistant population. Percentage similarity is given on the scale above the dendrogram. Subtypes of a genotype are designated a number after the genotype letter.

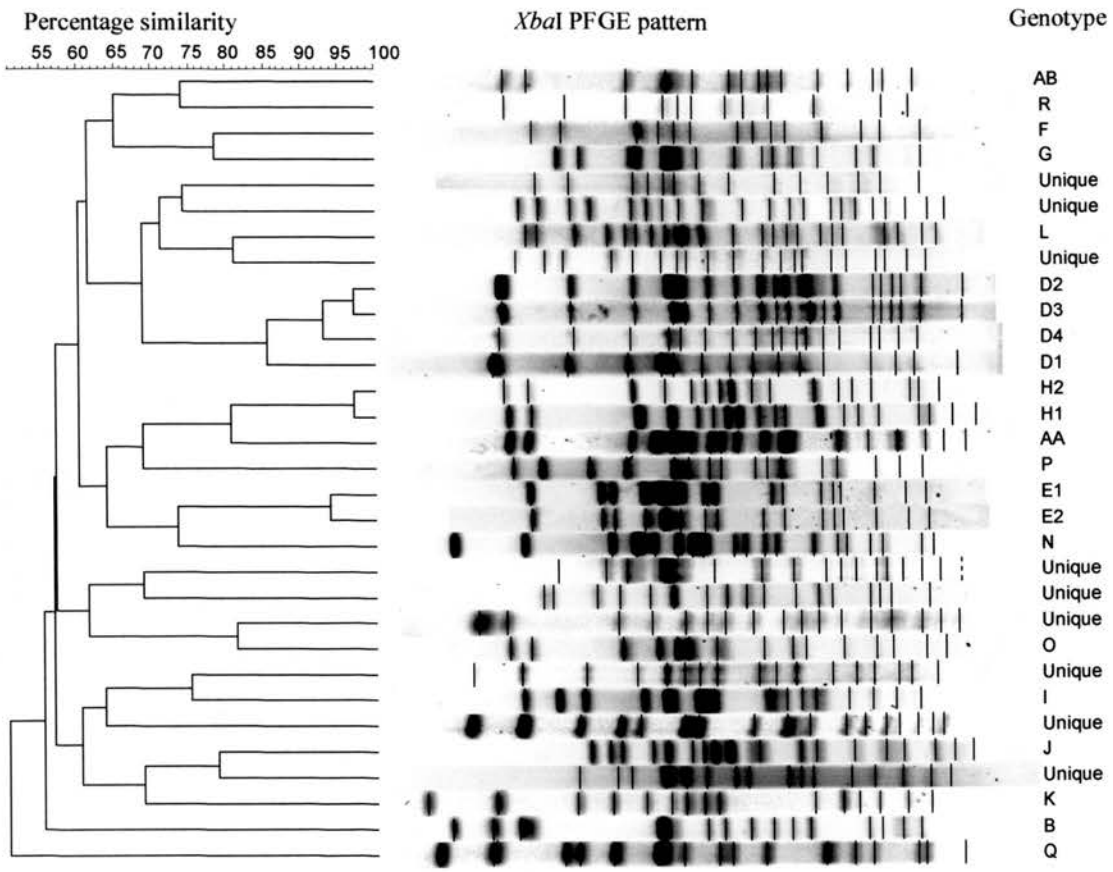


Figure 4.1c PFGE types in the ampicillin resistant population. Percentage similarity is given on the scale above the dendrogram. Subtypes of a genotype are designated a number after the genotype letter.

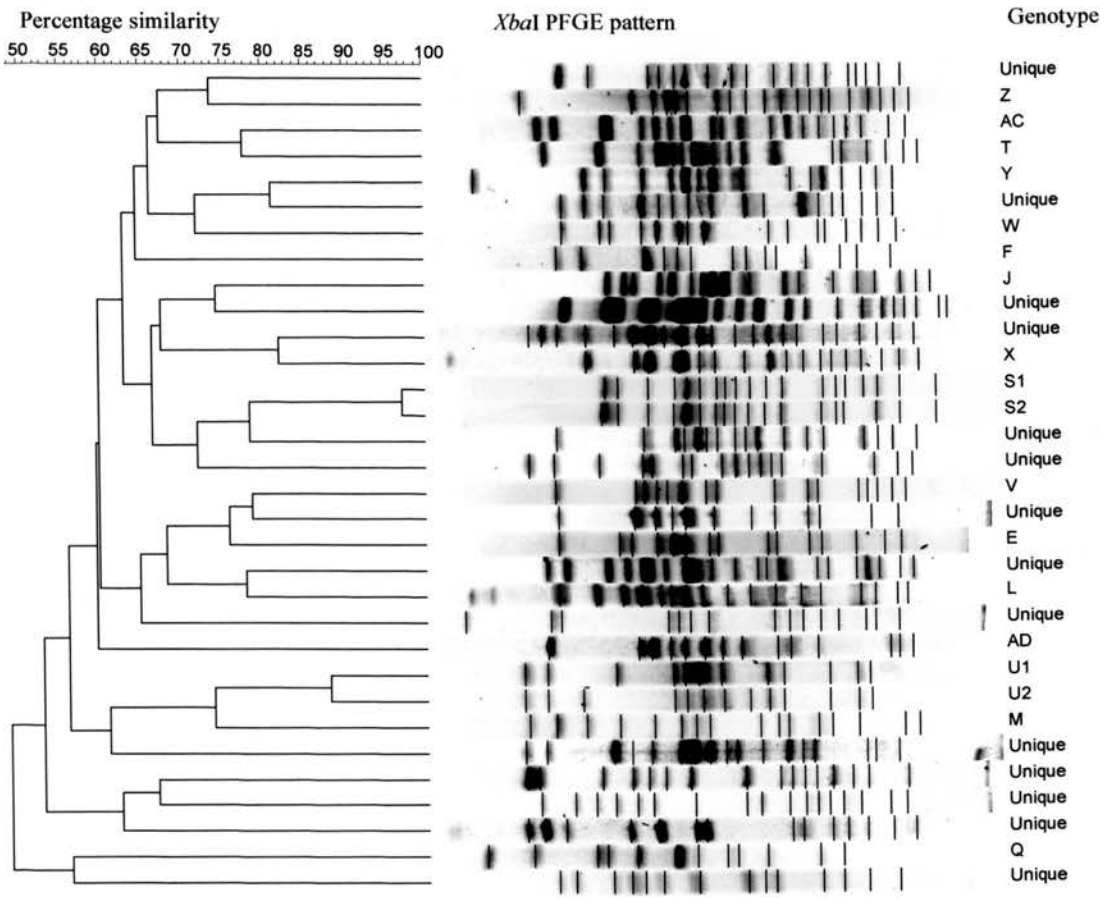


Figure 4.1d PFGE types in the unselected population. Percentage similarity is given on the scale above the dendrogram. Subtypes of a genotype are designated a number after the genotype letter.

The highest number of genotypes is found within the unselected population (30 genotypes, two of which contained subtypes) (Figure 4.1d). Of the selected populations, the amp^R population contained the most genotypes (26 genotypes) (Figure 4.1c), and the nal^R population the least (two genotypes) (Figure 4.1b). Five genotypes were found in the apr^R population (Figure 4.1a). In total 55 genotypes were detected within the 543 commensal *E. coli* examined in this study.

Within a given population, diversity may be expressed not only as the number of genotypes detected but also as the relatedness between genotypes. To compare the clustering patterns of each population (excluding the nal^R population as it only contains two genotypes), the numbers of lineages were plotted against increasing percentage similarity (Figure 4.2) as calculated by the dice coefficient. These plots are similar to lineage-per-time plots (Martin, 2002) except that percentage similarity rather than arbitrary time since common ancestry is plotted on the x-axis.

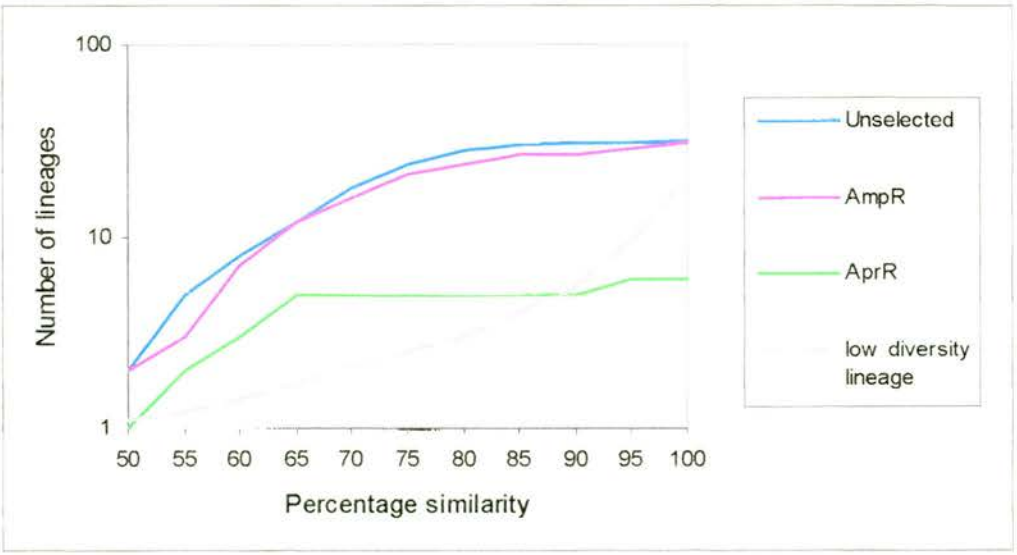


Figure 4.2 Numbers of lineages in the unselected, amp^R, and apr^R populations plotted against increasing percentage similarity as calculated by the dice coefficient. A hypothetical low diversity lineage is plotted in grey dashes as a comparison to these populations.

Figure 4.2 compares the relatedness of the unselected, amp^R and apr^R lineages. A dendrogram in which there are many closely related lineages would produce a convex curve as shown by the example lineage in Figure 4.2. A concave curve is produced when a large amount of diversity between lineages is present near to the root of the dendrogram. All three populations have concave curves, and there is very little difference between the unselected and the amp^R curves. One curve is not obviously more concave than the other. This indicates that the diversity between the different genotypes within these two populations is very similar. In support of this, the maximum percentage difference found between genotypes of the unselected and amp^R populations were 52% and 51% respectively.

The diversity within the unselected, amp^R, apr^R and nal^R populations were further explored using rank abundance curves. Figure 4.3 shows the total diversity found across all four populations. The curves for each population are shown in Figure 4.4.

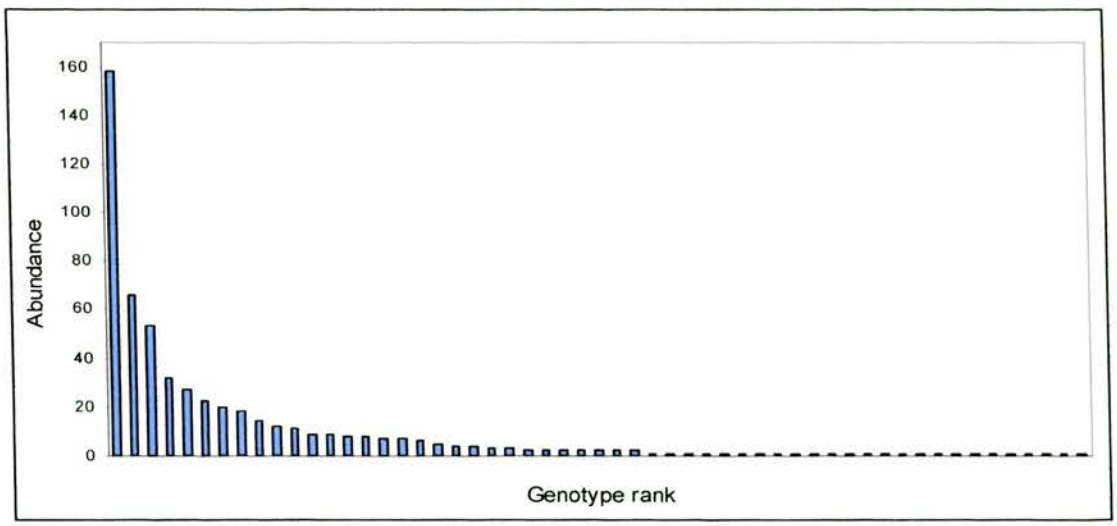


Figure 4.3 Rank abundance curve of the 543 commensal *E. coli* genotyped by PFGE in this study.

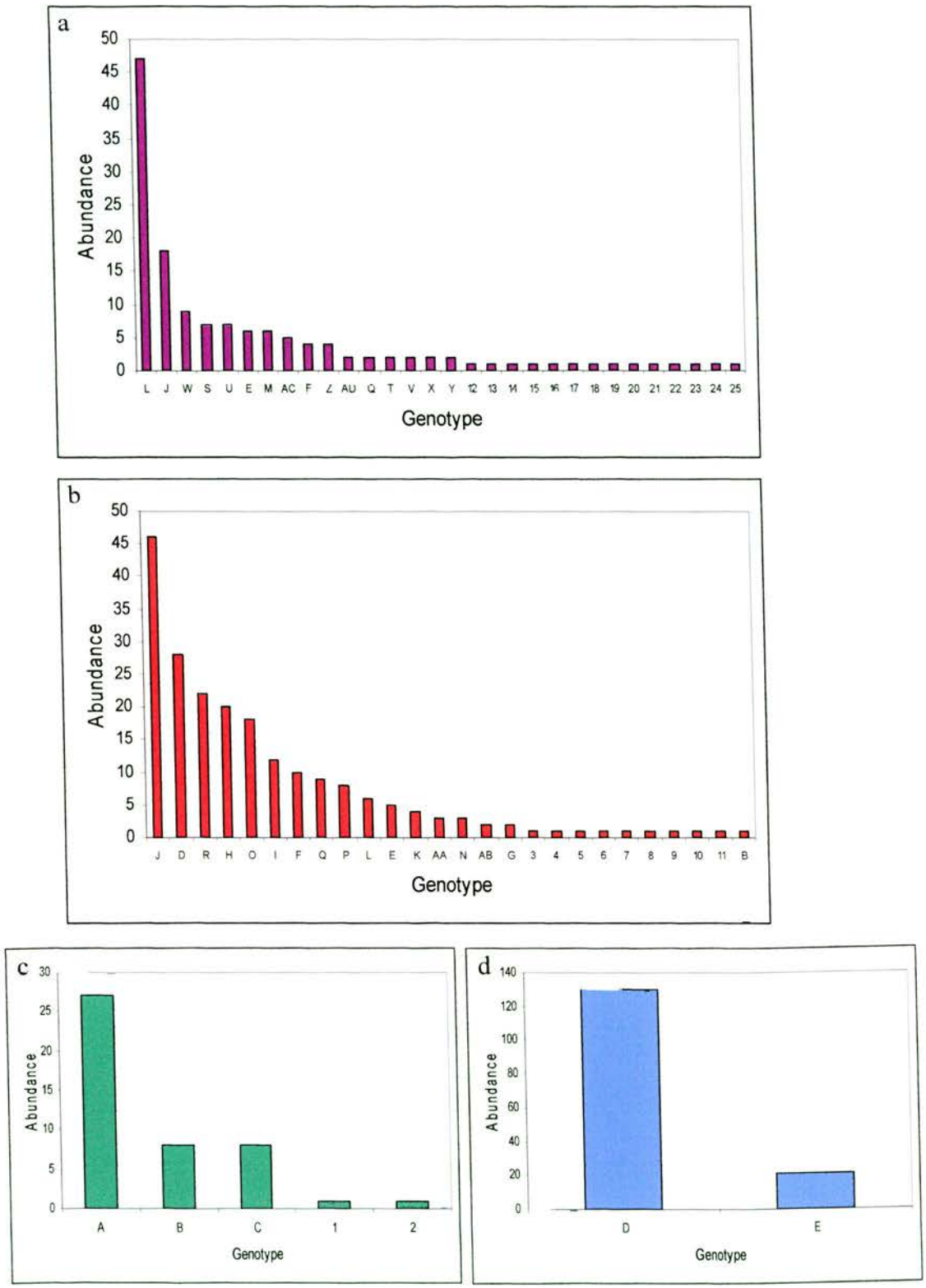


Figure 4.4 Rank abundance curves of a; the unselected population, b; amp^R, c; apr^R, and d; nal^R populations.

The rank abundance of each genotype, irrespective of the population from which it was obtained, is shown in Figure 4.3. Although the data may be biased by the use of different numbers of multiple isolates from each faecal sample, the shape of this rank abundance curve is typical of a superdiverse community, as is expected. In Figure 4.3, it can be seen that population contains a few abundant genotypes and many rare genotypes. This produces a long right-hand tail on the rank abundance curve. Comparing the curve of the unselected population (Figure 4.4a) to that of the amp^R population (Figure 4.4b) reveals very little difference between them. In terms of richness, the amp^R population has fewer genotypes, but the evenness of spread of genotypes between the amp^R and the unselected population are very similar. The diversity of the amp^R population is substantially greater than that of the apr^R and nal^R populations (Figure 4.4c and d).

Finally, the diversity within each of the four populations was compared qualitatively using diversity indices. The two diversity measures used evaluate different aspects of the term diversity. Chao1 is a species richness estimate, whereas Simpson's diversity index (C) measures the probability of finding two identical genotypes when two isolates are taken at random from a population. Table 4.2 shows the diversity of the four populations according to these diversity measures. 1-Simpson's index (designated D), is given in Table 4.2, so that D increases with increasing diversity.

Table 4.2 Diversity measures of the nal^R, apr^R, amp^R and unselected populations.

Population	No. of isolates	No. of genotypes found	Diversity measure	
			Chao1 (richness estimate)	1-Simpson's (D) (diversity index)
Nal^R	151	2	2	0.241
Apr^R	45	5	9	0.592
Amp^R	208	26	49	0.899
Unselected	139	30	46	0.858

The species richness estimate Chao1 predicts that the least number of genotypes will be found in the nal^R population (Table 4.2). This is quite reasonable because in *E. coli* resistance to nalidixic acid commonly occurs by mutation in the quinolone

resistance determining region of the *gyrA* gene (Vila *et al.*, 1994). This is not a transferable resistance mechanism and so the number of genotypes harbouring this resistance gene are likely to be less than if it was carried on a transferable element (as are the apramycin and ampicillin resistance determinants). The amp^R population is predicted to have the most number of genotypes (n = 49), but it is unclear whether the differences in diversity or richness of the amp^R and the unselected population are significant (Table 4.2). Similarly, Simpson's diversity index also predicts the amp^R population to be the most diverse. Qualitatively, the two diversity measures predict the same trends, with the nal^R population being the least diverse and the amp^R, the most.

To explore the robustness of the Chao1 richness estimate, one fifth of the isolates from each population were randomly excluded from the analysis. Identical richness estimates were produced with these reduced data sets of the nal^R and apr^R populations, but not with the amp^R or unselected populations. The reduced amp^R population was predicted to contain 35 genotypes (as opposed to 49, see Table 4.2), and the reduced unselected population was estimated to comprise 48 genotypes (rather than 46). Therefore, smaller sample sizes had the greatest effect on the most diverse populations, and much less of an effect on lower diversity populations.

4.3b Genotype diversity

Isolates within 24 of the 55 genotypes had identical band patterns following digestion with *Xba*I. These genotypes and the populations from which they were obtained are summarised in Table 4.3.

Table 4.3 Genotypes found within the calf commensal *E. coli* for which no genotypic diversity was found following PFGE typing with *Xba*I.

Genotype	No. in genotype	Populations (No. found)
B	9	apr (8), amp (1)
C	8	apr
F	14	plain(4), amp (10)
G	2	amp
I	12	amp
J	64	amp (46), plain (18)
K	4	amp
L	53	amp (6), plain (47)
M	6	plain
N	3	amp
O	18	amp
P	8	amp
Q	11	amp (9), plain (2)
R	22	amp
T	2	plain
V	2	plain
W	9	plain
X	2	plain
Y	2	plain
Z	4	plain
AA	3	amp
AB	2	amp
AC	5	plain
AD	2	plain
Uniques	1 per group	apr (2), amp (9), plain (14)

Diversity within a genotype was found in six genotypes. These genotypes were split into subtypes designated by the genotype letter then the number of the subtype. The subtypes are numbered sequentially according to the number of isolates within them. Thus, subtype 1 of a genotype contains the majority of isolates within this genotype. In Table 4.4 these genotypes and subtypes are summarised. The number of isolates within a subtype is given, in addition to the band changes of these isolates compared to the majority band pattern of the genotype.

Table 4.4 Genotypes found within the calf commensal *E. coli* for which diversity within a type was found following PFGE typing with *Xba*I. The populations from which these isolates were obtained are listed in addition to the band changes of these isolates compared to the majority band pattern of the genotype.

Genotype	No. in genotype	Populations (No. of isolates detected)	Subtype	Band differences from majority subtype	No. of isolates
A	27	apr (27)	A1	NA	25
			A2	531bp replaced by 563bp	2
D	158	nal (130), amp (28)	D1	NA	102
			D2	106bp missing	47
			D3	94bp missing	7
			D4	34bp missing	2
E	32	nal (21), amp (5), plain (6)	E1	NA	19
			E2	151bp missing	10
			E3	151bp replaced by 159bp	3
H	20	amp (20)	H1	NA	19
			H2	139bp missing	1
S	7	plain	S1	NA	6
			S2	57bp band extra	1
U	7	plain	U1	NA	6
			U2	355bp replaced by 418bp, 172bp band extra	1

NA, these are the majority genotypes against which the subgroup patterns were compared.

Within the six genotypes listed in Table 4.4, all but one subtype differed from the majority subtype by one band difference (i.e. 1 band shifted, missing or extra). The subtype of genotype U (U2) had a two-band difference from the majority subtype U1.

4.3c Within-sample diversity

The diversity of genotypes present within one sample was examined with each population. The number of genotypes detected as a function of the number of isolates typed is presented in Figures 4.5a-d.

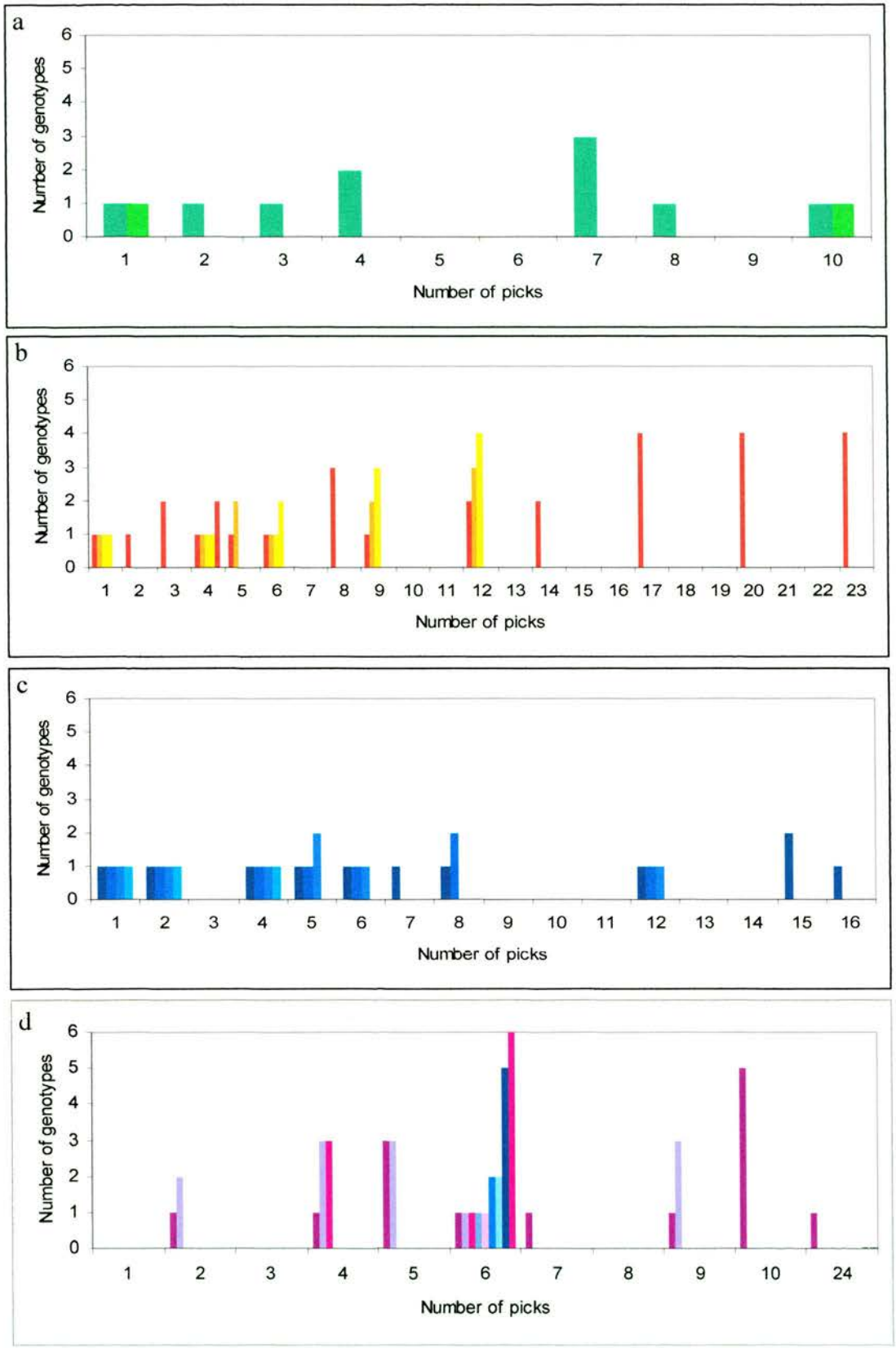


Figure 4.5 Within-sample diversity presented as the number of genotypes detected within a sample from the a; apr^R, b; amp^R, c; nal^R, and d; unselected populations.

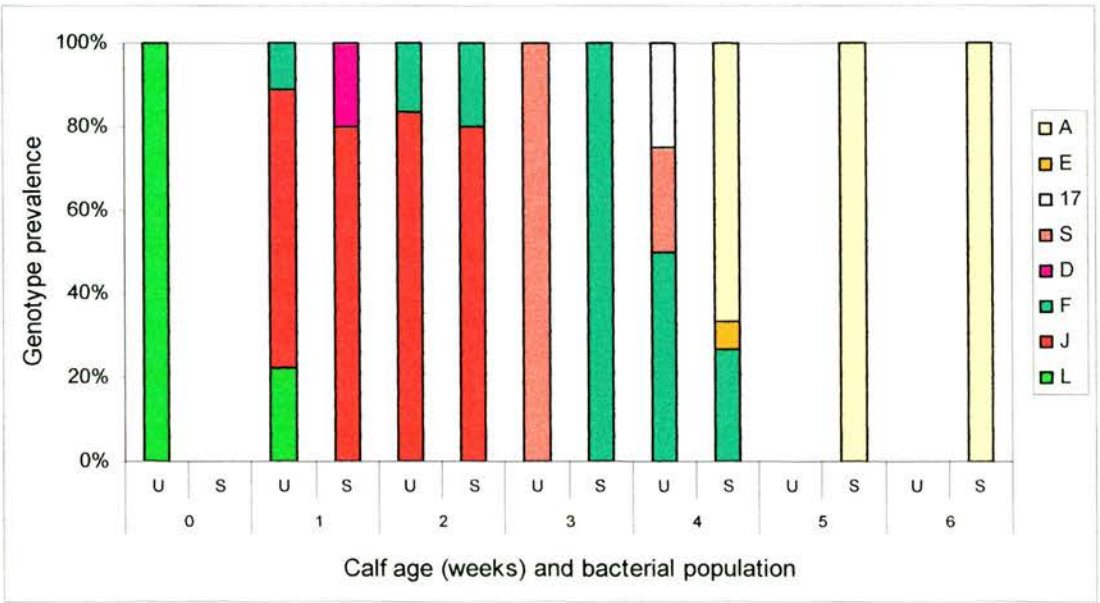
The number of isolates obtained from a sample varied because the criterion for picking colonies was to pick the maximum possible numbers of discrete colonies. In some samples many colonies were present, but in others either low numbers of colonies grew or those that were present were not discrete. Where the same numbers of picks were obtained from more than one sample, the different numbers of genotypes detected in each of these samples are plotted on the graphs in different shades.

When up to 10 colonies were picked from the apramycin plates, a maximum of only three different genotypes were detected (Figure 4.5a). When up to 16 colonies were obtained from a nalidixic acid containing plate, the maximum number of genotypes detected was only two (Figure 4.5c). A maximum of four different genotypes were detected amongst 12 colonies from an ampicillin plate (Figure 4.5b). These results are in contrast to those obtained for the unselective plates where six colonies on one plate contained six different genotypes (Figure 4.5d). The within-sample diversity appeared to level off at a maximum of four genotypes per sample in the amp^R population when up to 23 different colonies were obtained from a sample. In the unselected population, within sample diversity was not consistent. Only one genotype was found amongst 23 colonies from one sample, in contrast to the detection of a different genotype in all of six isolates obtained from another sample (Figure 4.5d).

4.3d Population diversity as a function of calf age

To determine if the number and types of commensal *E. coli* changed with calf age, genotypes from calves 687, 690, 693 and 694 were studied in more detail. These calves were selected for this analysis because they had a good spread of isolates over at least four consecutive sampling dates since birth. None of the calves received any direct antibiotic treatment during the sampling dates used in this analysis (see materials and methods for treatment record details). Figures 4.6, 4.7, 4.8 and 4.9 show the genotypes detected from calves 687, 690, 693 and 694 respectively. The

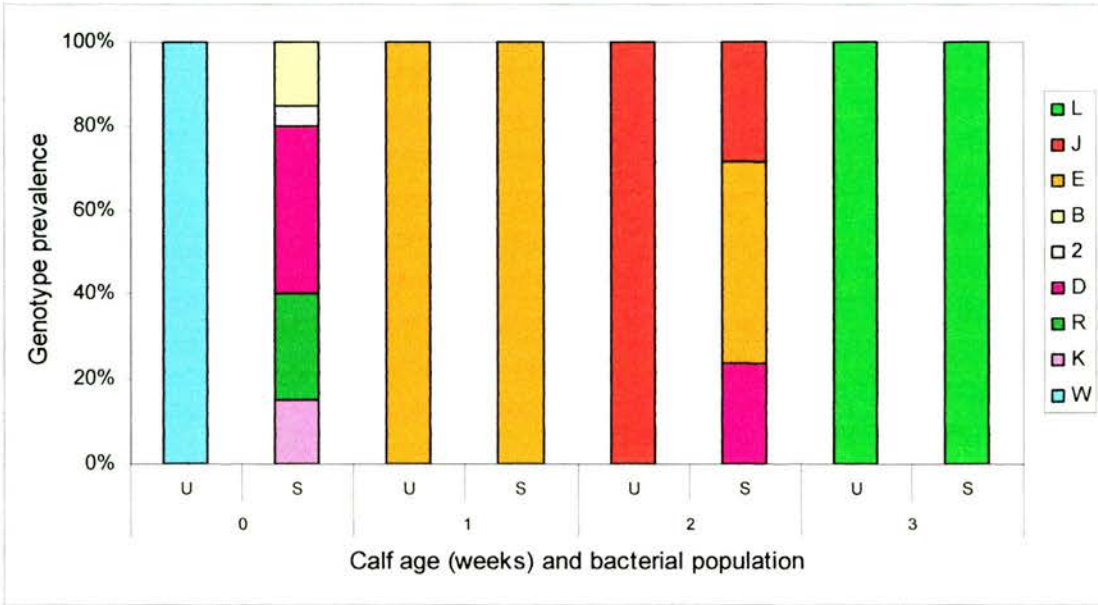
genotypes detected on each of the weeks after birth are given as a percentage of the total number of isolates obtained on that date. In each graph, the x-axis is split into two groups to present the genotype prevalence from the unselected (U) and selected (S) (comprising amp^R, apr^R and nal^R isolates) populations. All unique genotypes are shaded in grey. The other genotypes are indicated with colours that are consistent between the graphs. Below each figure is a table containing a breakdown of which genotypes were present in each of the unselected, amp^R, apr^R and nal^R populations, the age of the calf and the corresponding sampling date.



Date	Calf age (weeks)	Number of isolates (Genotypes detected)				
		Total	unselected	amp^R	apr^R	nal^R
17. 09. 01	0	24	24 (L)			
26. 09. 01	1	14	9 (L,J,F)	4 (J)		1 (D)
01.10. 01	2	11	6 (J,F)	5 (J,F)		
09. 10. 01	3	10	6 (S)	4 (F)		
17. 10. 01	4	19	4 (F,S,17)	4 (F)	10 (A)	1 (E)
24. 10. 01	5	10			10 (A)	
12. 11. 01	6	3			3 (A)	

Figure 4.6 and Table 4.5 Figure 4.6 shows the genotype prevalence with calf age of unselected (U) and selected (amp^R, apr^R and nal^R) (S) isolates obtained from calf 687. Table 4.5 lists the dates of detection, exact numbers of each genotype and populations from which they were isolated.

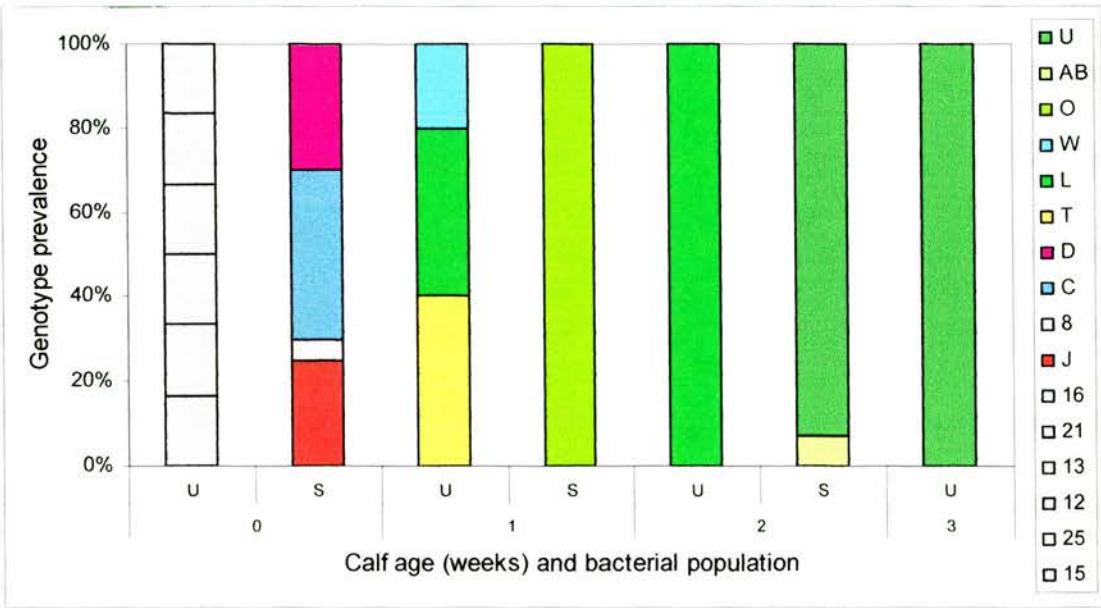
No resistant isolates were detected within 48hrs of birth of calf 687 (week 0, sampling date 17.09.02) (Figure 4.6). After one week, resistant isolates were detected. The amp^R genotypes J and F were detected in the selected population for at least two consecutive weeks. These genotypes were both present in the unselected population on more than one occasion, indicating a high prevalence of these strains. The nal^R isolate obtained in the first week was different to that obtained in the fourth week (D and E respectively). The waves of different colours across Figure 4.6 are indicative of different genotypes becoming the dominant type and then being replaced by a new dominant type on subsequent dates.



Date	Calf age (weeks)	Number of isolates (Genotypes detected)				
		Total	unselected	amp^R	apr^R	nal^R
26. 09. 01	0	26	6 (W)	9 (R,K,D)	4 (B,2)	7 (D)
01.10. 01	1	16	6 (E)	5 (E)		5 (E)
09. 10. 01	2	27	6 (J)	6 (J)		15 (D,E)
17. 10. 01	3	15	9 (L)	6 (L)		

Figure 4.7 and Table 4.6 Figure 4.7 shows the genotype prevalence with calf age of unselected (U) and selected (amp^R, apr^R and nal^R) (S) isolates obtained from calf 693. Table 4.6 lists the dates of detection, exact numbers of each genotype and populations from which they were isolated.

In contrast to calf 687, the most genotypic diversity within the commensal *E. coli* of calf 693 occurred in the first sample (taken within 48h of birth) (Figure 4.7). On this date, isolates were obtained from all four populations (unselected, amp^R, apr^R and nal^R). However, a week later (01.10.01), when isolates were obtained from the unselected, amp^R and nal^R populations, all isolates were found to be of the same genotype (E). The fact that this genotype accounted for all six of the isolates obtained from an unselective plate indicates that this strain occurred at a high prevalence on this date. This high prevalence of a strain resistant to ampicillin and nalidixic acid does not correlate with any antibiotic treatments of calf 693 (see Chapter 2).

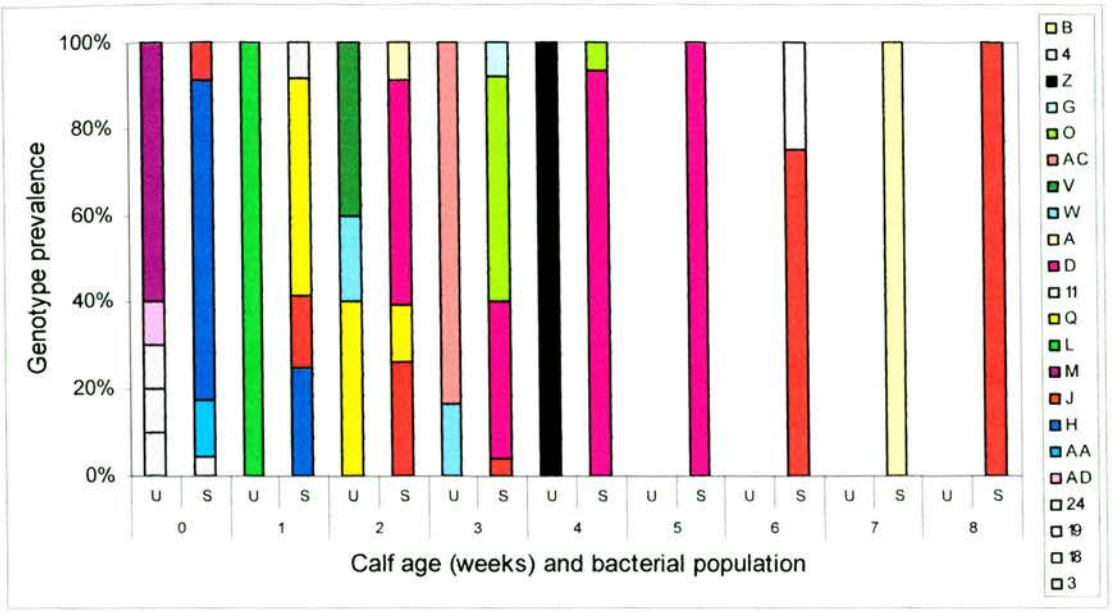


Date	Calf age (weeks)	Number of isolates (Genotypes detected)				
		Total	unselected	amp^R	apr^R	nal^R
26. 09. 01	0	26	6 (uniques)	6 (J,8)	8 (C)	6 (D)
01.10. 01	1	9	5 (T,L,W)	4 (O)		
09. 10. 01	2	34	6 (L)	12 (AB,D)		16 (D)
17. 10. 01	3	7	7 (U)			

Figure 4.8 and Table 4.7. Figure 4.8 shows the genotype prevalence with calf age of unselected (U) and selected (amp^R, apr^R and nal^R) (S) isolates obtained from calf 694. Table 4.7 lists the dates of detection, exact numbers of each genotype, and populations from which they were isolated.

Similar to calf 693, the greatest diversity of genotypes isolated from calf 694, is observed from the sample taken within 48h of birth (26.09.01). In this sample, isolates were obtained from unselective, ampicillin, apramycin and nalidixic acid plates. In contrast to calf 693, the unselected isolates from the week 0 sample of calf 694 were all unique genotypes (genotypes only ever detected once).

The genotype diversity of calf 694 appears to decrease with increasing calf age in both the selected and unselected populations. On the three dates when isolates were obtained from both these populations, the resistant genotypes were never present in the unselected population. This is in contrast to the detection of resistant genotypes in the unselected populations of calves 687 and 693, and indicates that calf 694 has overall a lower prevalence of resistant genotypes than calves 687 and 693.



Date	Calf age (weeks)	Number of isolates (Genotypes detected)				
		Total	unselected	amp^R	apr^R	nal^R
18. 09. 01	0	33	10 (24,18,19,AD,M)	23 (AA,J,H,3)		
26. 09. 01	1	14	2 (L)	12 (Q,J,H,11)		
01.10. 01	2	28	5 (V,Q,W)	9 (Q,5)	2 (A)	12 (D)
09. 10. 01	3	31	6 (AC,W)	20 (O,D,G,J)		5 (D)
17. 10. 01	4	19	4 (Z)	3 (O,D)		12 (D)
24. 10. 01	5	13		9 (D)		4 (D)
29. 10. 01	6	4		4 (4,J)		
12. 11. 01	7	1			1 (B)	
19. 11. 01	8	1		1 (J)		

Figure 4.9 and Table 4.8 Figure 4.9 shows the genotype prevalence with calf age of unselected (U) and selected (amp^R, apr^R and nal^R) (S) isolates obtained from calf 690. Table 4.8 lists the dates of detection, exact numbers of each genotype, and populations from which they were isolated.

Examination of the unselected population of calf 690 revealed that the greatest number of different genotypes is found in the first sample (18.09.01). There appears to be a general trend towards decreasing diversity by week four (17.10.01). This trend of decreasing diversity with increasing calf age was observed in samples obtained from calf 693. The amp^R population also appears to decrease in diversity with increasing age of calf 690.

Similar to calf 687, some genotypes appear on at least two consecutive sampling dates and then disappear below the detection threshold (≤ 1000cfu/mL) (e.g. H, D, O, W and Z). Genotype J is found within the amp^R population on six different sampling dates. J is never detected within the unselected population suggesting that it is present at a lower prevalence in calf 690 than in calf 693, where it was detected in the unselected population. In contrast to calves 693 and 687, resistant genotypes are only found in the unselected population on one occasion (01.10.01).

Overall, three genotypes (F, A and D) were found to persist for at least three consecutive weeks in a particular calf. Eight PFGE types persisted for two weeks (L, J, S, E, W, H, Q, and O). Forty-one genotypes (including unique types) were only found on one sampling date in a given calf. To determine which genotypes were detected in more than one calf, and which were unique to a calf, the presence or absence of each genotype was listed in a table (Table 4.9). Blue shading indicates the presence of a genotype, and no shading, the absence of a genotype. The unique isolates were not included in this table because they were only found on one occasion each, and so are by nature unique to each calf that they were isolated from (at the detection level of the method, ≤ 1000 cfu/mL).

Table 4.9 Presence (blue shading) or absence (no shading) of genotypes detected in calves 687, 693,694 and 690.

Genotype	Number of calves	Calf 687	693	694	690
D	4	■	■	■	■
J	4	■	■	■	■
L	4	■	■	■	■
W	3		■	■	■
A	2	■			■
E	2	■	■		
O	2			■	■
AA	1				■
AB	1			■	
AC	1				■
AD	1				■
B	1		■		
C	1			■	
F	1	■			
G	1				■
H	1				■
K	1		■		
M	1				■
Q	1				■
R	1		■		
S	1	■			
T	1			■	
U	1			■	
V	1				■
Z	1				■

Table 4.9 shows that genotypes D, J and L were detected in all four calves, but the majority of genotypes (18/25) were unique to the calf from which they were isolated.

To summarise the data presented in this section of work, the following observations of the data were made:

1. The most genotypic diversity was observed in calves 693, 694 and 690 in the first sample, and appeared to decrease with increasing calf age.
2. Only calf 687 did not harbour resistant isolates on the first sample.

3. Genotypes isolated from antibiotic containing plates were detected in the unselected populations of calves 693 and 687 (and 690 on one occasion) indicating a high prevalence of resistance in these calves.
4. Genotypes isolated from antibiotic containing plates were not detected in the unselected population of calf 694, indicating a lower prevalence of resistance than in calves 693 and 687.
5. The majority of genotypes were unique to the calf from which they were isolated, but genotypes D, J and L were detected in all four calves.
6. Genotypes F, A and D persisted for at least three weeks in at least one calf.

From the data presented in Figures and Tables 4.7 to 4.10, it was hypothesised that genotypic richness (number of types detected) decreases with calf age, and that the level of diversity was specific to a particular calf. When these hypotheses were tested with the data from all 11 calves, genotypic richness was found to decrease with increasing calf age ($F_{1,62} = 9.13$, $P = 0.004$), but was not associated with specific calves ($F_{10,52} = 1.59$, $P = 0.14$), sampling dates ($F_{1,61} = 2.63$, $P = 0.11$), or population (i.e. unselected or selected populations) ($F_{1,61} = 0.024$, $P = 0.877$). The rate of decrease in genotype richness detected with increasing calf age was dependant on the calf ($F_{8,43} = 3.20$, $P = 0.007$), but not the sampling date ($F_{1,51} = 0.55$, $P = 0.463$), or population (unselected versus selected) ($F_{1,42} = 0.46$, $P = 0.50$).

4.3e Genotype diversity as a function of sampling date

To explore the diversity amongst genotypes as a function of sampling date, genotypes that were detected on more than one sampling date (not necessarily the most abundant genotypes shown in Figure 4.4) were plotted against sampling date for the unselected, amp^R and nal^R populations (Figure 4.11, 4.12 and 4.13 respectively). The apr^R population is excluded from this analysis as it is discussed in detail in Chapter 5.

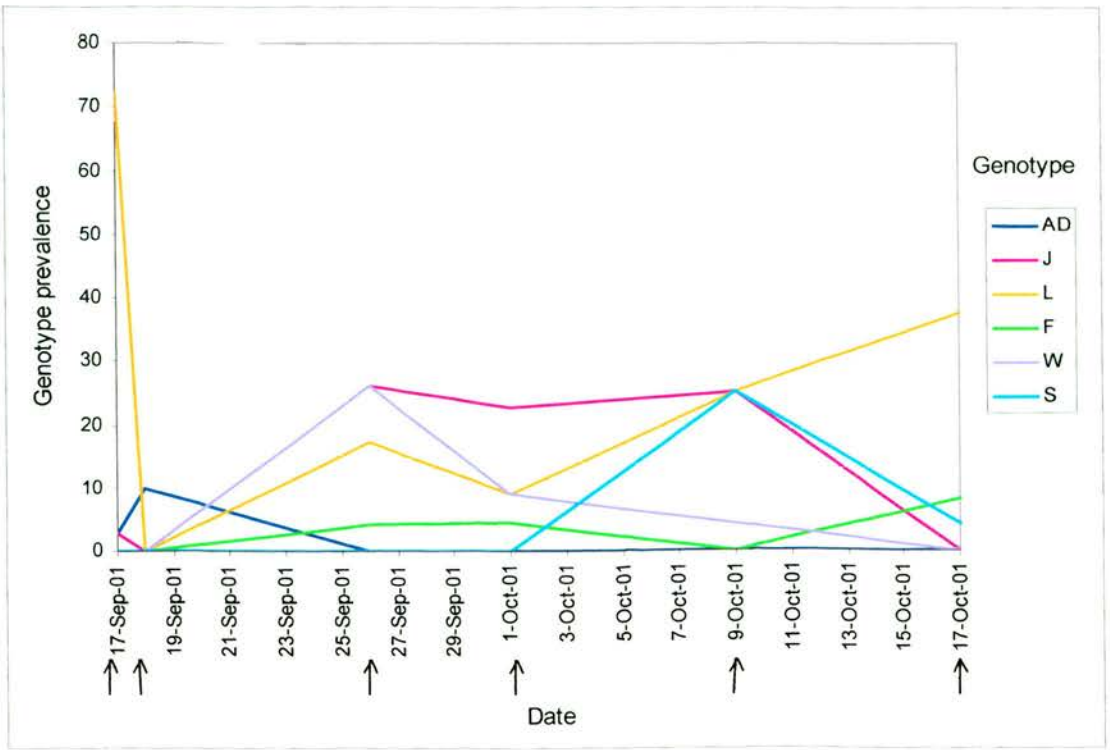


Figure 4.10 Genotype prevalence within the unselected population. Arrows indicate sampling dates.

The most prevalent genotype (L) on 17.09.01 is not detected the following day (18.09.01), instead AD is the most prevalent genotype (Figure 4.10). The samples obtained from 17.09.01 and 18.09.01 are from different calves, therefore are likely to reflect the differences in genotypes between these calves. Unfortunately, consecutive samples from these two dates from the same calf were not available. After 18.09.01, genotype L shows an overall increase in prevalence over the following 11 days. In contrast, genotypes W and J peak on 26.09.01 and then tend to decrease to undetectable levels on 17.10.01. These dynamics suggest that these more common genotypes prevail for a few days but do not seem to appear and disappear below detection threshold over the sampling period shown. The lack of samples between the dates when samples were obtained means that any trends observed in this graph are difficult to interpret with any certainty.

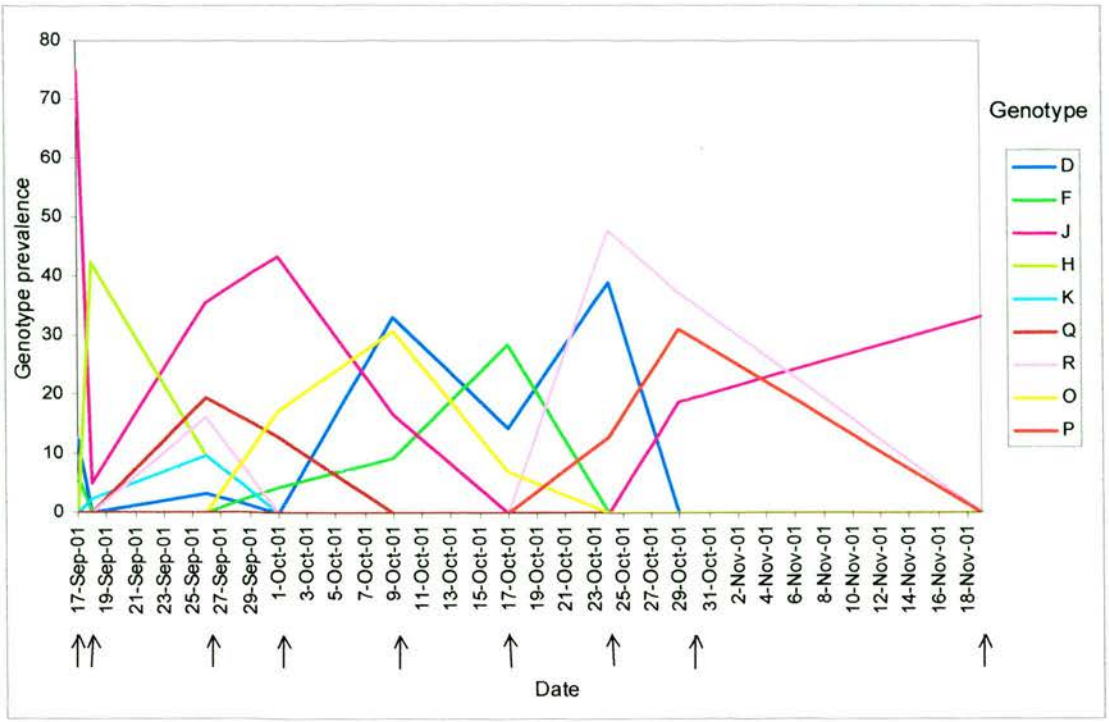


Figure 4.11 Genotype prevalence's within the amp^R population. Arrows indicate sampling dates.

The overall impression of the genotypes within the amp^R population is one of dramatic fluctuations (Figure 4.11). Gradual increases or decreases in genotype prevalence are rarely observed with this sampling protocol. Genotypes that appear to vary between being present and absent on different sampling dates are likely to be present at a prevalence that fluctuates around the detection threshold of the sampling and detection methods.

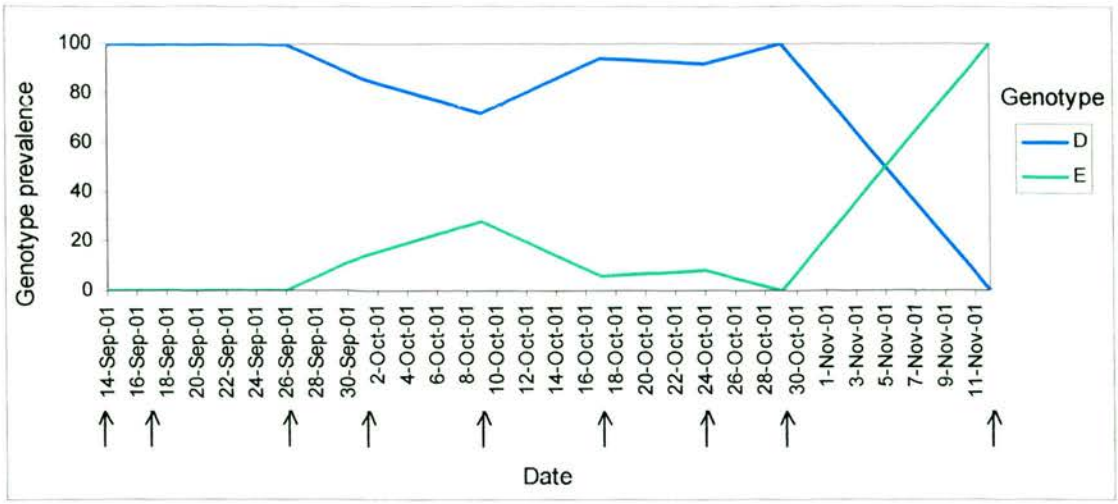


Figure 4.12 Genotype prevalence's within the nal^R population. Arrows indicate sampling dates.

The most common genotype in the nal^R population is D. On one sampling date (19.10.01) it was not detected, and type E is the only nal^R genotype found (Figure 4.12). There appears to be a switching of the most prevalent genotype between 29.10.01 and 19.11.01. To prove this hypothesis, further sampling would be required.

4.4 Discussion

The majority of studies of *E. coli* diversity have focused on pathogenic strains (Hartman *et al.*, 2003; Kerrn *et al.*, 2002; Noller *et al.*, 2003; Ørskov *et al.*, 1990; Shen *et al.*, 1999; Whittam *et al.*, 1993), but comparatively little is known about the population structure of commensal *E. coli*. It is essential to determine the background level of population diversity if valid conclusions are to be drawn as to the genetic relatedness of groups of isolates within the *E. coli* genus (for example outbreak strains, pathogenic strains or antibiotic resistant strains). This chapter aimed to study different aspects of the population diversity of commensal *E. coli* isolated from calves. Firstly, the genotype diversity within unselected populations and amp^R, apr^R and nal^R subpopulations was compared. Next, the diversity within genotype groups (subtypes of a genotype) and diversity of genotypes within a faecal sample was

examined. Finally, changes in the diversity of genotypes with calf age or with sampling date were explored.

Before each of these sections of work is discussed, it is important to ascertain exactly what is meant by the term diversity. Diversity may be expressed in three different ways; the number of types of an organism (i.e. species richness), the evenness or relative abundance of each type of organism, and the relatedness between groups of organisms. There is no single measure capable of expressing each of these aspects of diversity within a population. In this chapter, a combination of different approaches was used to compare the genotype diversity of the unselected, amp^R, apr^R and nal^R populations.

4.4a Population diversity

Counting the number of genotypes detected within the unselected, amp^R, apr^R and nal^R populations revealed the unselected population to have the most number of genotypes, and the nal^R population to have the least. Only four more genotypes were found in the unselected population compared to the amp^R population, indicating a high degree of diversity between strains resistant to the β-lactam ampicillin. Although plots of the number of lineages versus percentage similarity demonstrated the genetic relatedness (or dissimilarity) between genotypes within these two populations to be very similar, the spread of genotype abundance within the unselected population was less even, therefore showed less diversity than the amp^R population. This emphasises the need to use different measures of diversity in any assessment of diversity between two populations. Each measure on its own does not provide all the information required to assess the three aspects of diversity described at the start of this discussion. In agreement with this, Bohannan & Hughes (2003) also recommend the use of a combination of different approaches to analyse diversity in microbial communities.

Similar to the high level of diversity found within the amp^R population, Shen *et al.* (1999) describe a wide genetic diversity amongst clinical isolates of *E. coli* producing extended-spectrum β-lactamases (eight different PFGE patterns were obtained from only 23 isolates). In the comparison of the four populations described in this chapter, the nal^R population was the least diverse. Similarly, fluoroquinolone resistant *E.coli* from cancer patients were clonal within each patient (Oethinger *et al.*, 1998).

The reason for the different degrees of diversity within the resistant populations studied is likely to be because of the mechanism of resistance harboured. Resistance to β-lactam antibiotics is commonly plasmid-mediated (Datta & Kontomichalou, 1965; Heritage *et al.*, 1999; Philippon *et al.*, 2002). In addition to the ability to develop resistance individually by mutations in genes controlling AmpC production, resistance may be acquired by the acceptance of a resistance plasmid. A detailed analysis of the diversity of the plasmids conferring amp^R would confirm this. In contrast, until recently resistance to the quinolones was never considered to be plasmid mediated (Amyes *et al.*, 1996), however evidence of a plasmid vector has been found in isolates from China (Wang *et al.*, 2003). Therefore, the most likely way for a strain to acquire resistance is by mutation of the quinolone resistance determining region of the DNA gyrase and topoisomerase II genes.

Although apramycin resistance is also plasmid-mediated, the genotypic diversity within the apr^R population is not as great as in the amp^R population. It is likely that amp^R plasmids are more common because β-lactams are used more frequently on the farm than aminoglycosides (see Chapter 2). Another factor affecting the spread of plasmid-mediated resistance is plasmid host range. Are the amp^R plasmids broad host range plasmids and the apr^R plasmids of a narrow host range? Some results on the acceptance of apr^R plasmids are presented in Chapter 5.

Various diversity estimates have been used previously to compare bacterial diversity between different environments (Dunbar *et al.*, 1999; McCaig *et al.*, 1999; Sigler & Zeyer, 2002). Simpson's diversity index measures the probability that two

individuals from a population will be the same if the isolates are drawn at random. This was considered an appropriate measure because it expresses the dominance of one or two of the commonest species (or genotypes). The sampling protocol used in this work was suitable for determining the commonest genotypes. In contrast, the commonly used Shannon-Weaver diversity index is strongly affected by the abundance of the genotypes present in the middle of a range of genotype abundances. Although this measure has been applied to microbial populations (Dunbar *et al.*, 1999; McCaig *et al.*, 1999; Sigler & Zeyer, 2002) it was considered inappropriate for the bacterial populations described in this chapter because they contained a few common genotypes and many rare ones (as demonstrated by the rank abundance curves). The Shannon-Weaver diversity index assumes that the random sample taken from the population represents all of the species in the population. This assumption could not be even partly satisfied with this data set because of the extreme diversity of the commensal *E. coli* population (see Figure 4.3) and the sampling protocol adopted in this study.

Simpson's diversity index (D) ranges from 0 to 1. Values around 0 reflect the dominance of a few genotypes, and values nearer 1 reflect a population containing many different genotypes. The low D observed with the nal^R resistant population (D = 0.241) demonstrates the high dominance of a few genotypes. In contrast the diversity of the amp^R population was high (D = 0.899). This level of diversity is comparable to that found amongst *E. coli* strains in piglets within the first week of birth (mean D = 0.848) (Katouli *et al.*, 1995).

Qualitatively, diversity as estimated by Simpson's was similar to the diversity (defined as species richness) estimated by Chao1. Of 139 unselected *E. coli* comprising 30 different genotypes, total genotype richness was estimated at 46 genotypes. This is substantially lower than the number of *E. coli* predicted (predicted n = 128) with the same richness estimate amongst 280 *E. coli* from a bovine feedlot (Yang *et al.*, 2004). However, the number of types detected will depend on the typing method, the definition of a type, and the sampling effort. In the study of bovine feedlot *E. coli*, PCR-based typing was performed and operational taxonomic

units (OTUs) were defined with a cut-off of 85% similarity. Differences in methodology and analysis mean it is difficult to compare diversity between studies.

Interestingly, Seurinck *et al.* (2003) conclude that generally 32 isolates per faecal source were sufficient to characterise the richness of the *E. coli* within that source. In the present study, a maximum of 24 isolates were obtained from any one faecal sample, and so this hypothesis could not be challenged. However, bacterial population dynamics are based on the ability to persist at low levels and then increase when the selective environment favours the particular strain. It is possible that there could be a dominant strain that is 100-fold more abundant than any of the others, particularly if the environment favoured it. The other strains at lower concentrations would remain undetected by Seurinck *et al*'s criteria.

There are two important problems associated with the use of statistical approaches to estimate bacterial diversity. Firstly, they rely on estimates of relative abundances of OTUs, which may be inaccurate because of sampling biases. In the present study, the different numbers of isolates analysed from each sample may have introduced some bias into the data. Bias may also occur because of differences in survival during storage at 4°C between *E. coli* genotypes, prior to analysis. Secondly, the statistical methods used in this chapter rely only on the information from the genotypes observed and therefore cannot account for very rare genotypes. Although this will result in an underestimate of the actual number of genotypes present within a sample, they do provide a lower boundary of genotype diversity.

4.4b Genotype diversity

Subtypes within a genotype were identified in six of the 55 genotypes detected in the commensal *E .coli*. The presence of subtypes within a genotype depends on the interpretive criteria used to define a genotype. Commonly, criteria outlined by Tenover *et al.* (1995) are used in defining PFGE types. The usefulness of these definitions depend on the type of bacteria studied (more genotypic diversity may be

expected between *Streptococcus milleri* group (Bartie *et al.*, 2000) than between enterococci isolates (Perlada *et al.*, 1997)), the size of geographical area from which isolates were obtained (isolates from different countries are likely to be less similar than isolates from the same town unless they are related) and the diversity expected between the isolates (diversity between *E. coli* O157 is less than between commensal *E. coli* isolates comprising many serogroups (Vali *et al.*, 2004)).

In this chapter, a high degree of diversity was expected between the commensal isolates because there was no (known) selection for specific serogroups. The genotypes detected were highly diverse, with an uncountable number of band differences between genotypes. Because the isolates were found to be so diverse, the presence of types that differed from a genotype by up to two bands were considered related to that genotype. Only by taking into account the background level of diversity within the commensal population could this judgment be made. Similarly, Foxman *et al.* (2002) established a high degree of diversity between *E. coli* from pairs of heterosexual partners, which meant that finding the same genotype within a couple was of significance. In this chapter, the criteria devised by Tenover *et al.* adequately distinguished between different genotypes, which were highly dissimilar, and subtypes within a genotype. The same interpretive criteria have been used to characterise extended-spectrum β-lactamase producing clinical *E. coli* (Shen *et al.*, 1999) and enterohaemorrhagic *E. coli* (Rios *et al.*, 1999).

4.4c Within sample diversity

Increasing the numbers of colonies taken from a sample did not necessarily increase the number of genotypes detected. In the amp^R population increases in genotype numbers appeared to level off at 12 picks (where a maximum of four genotypes were detected in samples from which 12 to 23 isolates were obtained). The maximum genotype richness in the unselected population was found when six isolates were obtained, and increasing the number of isolates from a sample did not increase the richness detected (up to 23 isolates per sample). Based on the shape of the rank

abundance curve in Figure 4.4a it is hypothesised that a far greater number of rarer genotypes would be detected were log fold increases in isolates examined. This hypothesis is supported by the fact that when selection is applied to the commensal population (i.e. plating onto ampicillin plates) almost as many different genotypes are observed as in the unselected population (Figure 4.1c and d).

There appears to be no published data describing genotypic diversity of commensal organisms within samples (faecal or other), although phenotypic diversity has been described with 12 *E. coli* isolates from piglet faeces (Katouli *et al.*, 1995). The detection of more than one genotype in over half of the samples from the unselected population (11/21) and in 19 of the 63 samples from the selected population has important implications for the design of experiments intended to study genotypic diversity in either antibiotic resistant or unselected bacterial populations.

4.4d Population diversity as a function of calf age

The genotypic diversity (number of types) was found to decrease significantly with calf age (irrespective of whether the selected or unselected population was studied). This result contrasts with that of Katouli *et al.* (1995), where the mean number of faecal *E. coli* biochemical phenotypes found in piglets increased as the animals aged. Similar to Katouli *et al*, most types were found to be transient and were replaced by new types in the following samples. Genotypes F, A and D were found to persist for at least three weeks. These genotypes may have been better able to colonise and maintain their populations in the calf intestine than the majority of genotypes (n = 44), which were not detected on consecutive sampling dates. Colicin resistance has been found in established strains of *E. coli* (Ofek *et al.*, 1977), which was thought to aid persistence in the lower intestine.

Alternatively, genotypes F, A and D, may simply have been the first to colonise the calves. Freter *et al.* (1983) describe difficulties in implanting *E. coli* in the mouse intestine when established intestinal flora are present. In contrast, when the *E. coli*

are inoculated first, then the caecal flora, stable *E. coli* populations persist indefinitely.

4.4e Population diversity as a function of sampling date

The changes in PFGE types (detected on more than one occasion) did not show any general trends during the sampling period. The sampling protocol of approximately weekly sampling was not frequent enough to determine how genotype prevalences changed with time. A general impression of dramatic fluctuations of genotype prevalence was observed with both the unselected and amp^R populations. Similarly successive waves of phenotypically different *E. coli* have been found to colonise the intestine of piglets (Katouli *et al.*, 1995), although the sampling protocol of this study was less frequent than that described in this chapter.

The most prevalent nal^R genotype (D) appeared to be succeeded by a different genotype (E), but because 14 days had elapsed between the sampling dates when D was detected at a high prevalence, and the sampling date when a high prevalence of E was found, the dynamics of this switching of types are not clear.

4.5 Concluding remarks

Problems arose in analysing the diversity based on genotype prevalence, because the numbers of isolates obtained form each sample ranged from one to 24. A better protocol would be to spread diluted samples evenly over each agar plate, and to pick equal numbers of colonies from each plate showing growth. In addition, weekly sampling was found to be inadequate for studying the fluctuations of different genotypes with time. More frequent sampling (at least daily) is suggested for future analyses.

In this chapter a high degree of diversity amongst calf faecal commensal *E. coli*, in terms of the total number of different genotypes detected, the evenness of spread of types, and the genetic relatedness of genotypes was detected. Referring back to the original aims of the chapter, this work demonstrated the following:

1. Different genotypes were hypothesised to be present within one faecal sample. This was shown to be true in many but not all samples (11/21 unselected samples, 19/63 selected samples).
2. The statistical predictions of diversity produced qualitatively reasonable estimates, although the predicted numbers of genotypes are likely to be underestimates of the actual numbers present because they rely only on the number of genotypes observed. In this way, very rare classes are ignored because they are present below the detection threshold of the method. Rare classes may only be "rare" at the time of sampling because genotypes were found to fluctuate dramatically with time. On another sampling date, or following a change in feed, environment, housing or treatment, rarer types may be selected into becoming dominant types.
3. It was hypothesised that population diversity would differ between the resistant populations because the mechanisms by which bacteria may acquire resistance is different. The diversity observed differed between the four

populations, but the diversity between the unselected and amp^R populations were surprisingly similar. This was thought to be because ampicillin resistance is commonly plasmid mediated. The greater number of amp^R genotypes compared to the apr^R genotypes may reflect differences in the number and host range of these two types of resistance plasmid. In addition, the number of resistance genes that confer ampicillin resistance are numerous (Amyes *et al.*, 1996), in contrast to the single gene found to confer apramycin resistance (Shaw *et al.*, 1993).

4. The *E. coli* population diversity was hypothesised to decrease with calf age because the first colonisers, which did not have to compete for space and nutrients, would be out competed by genotypes better able to adapt to a more crowded and resource limited environment. Diversity (number of genotypes) was found to decrease significantly with increasing calf age, but was not significantly associated with sampling date.

Chapter 5. Molecular mechanism of apramycin resistance

5.1 Introduction

In 1978 the aminoglycoside apramycin was licensed for veterinary use in the United Kingdom, and has since been used extensively in animal husbandry (Hunter *et al.*, 1992). Although this antibiotic has not been used in human medicine, apramycin resistance has been detected in human isolates of *Salmonella typhimurium* (Chaslus-Dancla *et al.*, 1989), *Klebsiella pneumoniae* (Johnson *et al.*, 1995) and *E. coli* (Johnson *et al.*, 1995; Hunter *et al.*, 1993). Resistance to apramycin is conferred by the production of the aminoglycoside-modifying enzyme, 3-*N*-aminoglycoside acetyltransferase type IV (AAC(3)IV) (Davies & O'Connor, 1978) which has been found on diverse conjugative plasmids (Platt & Smith, 1991; Pohl *et al.*, 1993). A high degree of genetic homology has been detected between plasmids harbouring AAC(3)IV isolated from bacteria of human and animal origin (Chaslus-Dancla *et al.*, 1991). In addition to the acetylation of apramycin, AAC(3)IV also acetylates tobramycin and gentamicin, which are used to treat serious infections in humans (Amyes *et al.*, 1996). Recently, 7% of *E. coli* isolated from calf enteritidis faecal samples in Scotland were found to be resistant to apramycin (Gunn & Low, 2003). The aim of this study was firstly to determine whether calves that had not been treated with aminoglycosides harboured apramycin resistant (apr^R) commensal *E. coli,* and secondly to study the molecular epidemiology of the resistance genes to this antibiotic.

5.2 Materials and Methods

5.2a Bacterial isolates

Apramycin resistant *E. coli* isolates (n = 45) obtained as described in chapter 2 were examined in this study.

5.2b Antibiotic sensitivity

Minimum inhibitory concentrations (MICs) of ampicillin, apramycin, gentamicin, streptomycin, sulphamethoxazole, tetracycline (Sigma, Poole, UK) and tobramycin (Faulding Pharmaceuticals, Warwickshire, UK) were determined using a multipoint inoculator to replicate test cultures onto antibiotic-containing Isosensitest agar plates (Oxoid CM471) at 10^4-10^5 cfu/spot, following BSAC guidelines (Andrews, 2001). The MIC was defined as the lowest concentration of antibiotic to give complete inhibition of bacterial growth after incubation at 37°C for 18 hours.

Control organisms (NCTC10418, NCTC10662 and NCTC6571) were included to detect any errors in the preparation of antibiotics or plates, and all isolates were inoculated onto agar plates containing no antibiotic to ensure that any inhibition of growth on test plates was due to the presence of the antibiotic.

5.2c Pulsed-field gel electrophoresis

PFGE typing as described in Chapter 4 was used in this study.

5.2d Conjugation experiments

Apramycin resistance plasmids were transferred by conjugation into a rifampicin resistant *E. coli* J62-2 (pro$^-$ his$^-$ trp$^-$) (Bachmann, 1972) by incubating a 1:100 ratio of donor to recipient, in nutrient broth (Oxoid) at 37°C for 7 hours (method adapted from Provence & Curtiss, 1994). Transconjugants were isolated and purified on MacConkey plates (Oxoid) incorporating 20mg/L rifampicin and 16mg/L apramycin.

Transfer frequencies, completed in triplicate, were defined as the proportion of transconjugants over total number of donors (D) at the start of mating (Provence &

Curtiss, 1994) and were measured between J62-2 (D) and the related auxotroph J53 (pro⁻ met⁻) (Bachmann, 1972). Overnight nutrient broth (NB) (Oxoid) cultures of donor and recipient were incubated in NB at 37°C in a 1:100 ratio respectively, for 7 hours (Provence & Curtiss, 1994). Transconjugants were selected on Davis Mingioli plates (DM) supplemented with 16mg/L apramycin, 50mg/L proline and 50mg/L methionine. Average transfer frequencies were compared to those of the well-characterised plasmids RP4 (Datta *et al.*, 1971) and R46 (Brown & Willetts, 1981) using the same mating protocol.

The recipient ability of 28 different genotypes of commensal *E. coli* from the unselected population was measured by repeat conjugation experiments with J62-2 harbouring plasmid pUK2001. The same 7 hr mating protocol was used as that described above. Transconjugants were selected on DM plates containing no amino acids and 16mg/L apramycin.

5.2e Plasmid analysis

Plasmid DNA was extracted from J53 transconjugants using a Maxiprep kit (Qiagen) and restricted with *Eco*RI and *Apa*I (Promega) according to the manufacturer's instructions. Gels were stained with ethidium bromide as described earlier. Plasmid size estimates were determined by comparison to the standard plasmids harboured by *E. coli* 39R861 and *E. coli* V517.

5.2f Amplification of the aac(3)IV gene

Primers 5'-GTCGTCCAATACGAATGGCG-3' and 5'-CAGCAATCAGCGCGACCTTG-3' were used to amplify the region between base pairs 241 to 1076 of the published *aac(3)IV* gene (GenBank X01385) (Bräu *et al*, 1984) from purified plasmid DNA. DNA was denatured for 5 min at 94°C, followed by 30 cycles of 1 min denaturation (94°C), 1 min anneal (55°C), and 1 min extension

(72°C). Sequencing was performed by chain termination (Sanger *et al.*, 1977) in both directions and compared to the published sequence (Bräu *et al.*, 1984).

5.2g Statistical analysis

Statistical analysis was performed with S-Plus (Insightful, Seattle). Analyses of the carriage of apramycin resistant *E. coli* (presence/absence) by the 11 calves was performed using generalised linear mixed effects models, with binomial errors. Calf identity was entered as a random effect to account for both inherent differences between calves and the lack of independence between samples. Time and calf age were entered into separate models as fixed effects.

5.3 Results

5.3a Detection of apr^R E. coli PFGE types

Although no aminoglycoside antibiotics were used on the 11 calves sampled in this study, six calves harboured apr^R *E. coli* (≥1000 cfu/mL) during the four months sampling period. Detection dates, calf identities, antibiotic treatment records and genotypes of apr^R *E. coli* are listed in Table 5.1.

Table 5.1 Calves from which apr^R *E. coli* were isolated, calf antibiotic treatments and age, dates of apr^R *E. coli* detection, and number and genotype of apr^R isolates.

Calf	Treatment (date)	Sampling dates of apr^R *E.coli* detection	Apr^R *E. coli* isolates	PFGE types (number of each type)
690	None	01/10/2001	2	A (2)
		12/11/2001	1	B (1)
687	None	17/10/2001	10	A (10)
		24/10/2001	10	A (10)
		12/11/2001	3	A (3)
693	tylosin (21/11/2001) florfenicol (23/11/2001) florfenicol (05/12/2001)	26/09/2001	4	B (3), E (1)
694	None	26/09/2001	8	C (8)
696	None	12/11/2001	6	A (1), B(4), D (1)
698	enrofloxacin (01/10/2001)	10/12/2001	1	A (1)

From four calves (693, 694, 696 and 698), apr^R *E. coli* were found on only one occasion (Table 5.1). The analysis of carriage of apr^R *E. coli* by the 11 calves revealed that the detection of apr^R *E. coli* was not related to calf age ($F_{1,139} = 1.26$, $P = 0.26$), or sample date ($F_{1,139} = 1.22$, $P = 0.27$). To ensure that those calves for which apr^R *E. coli* were never detected were not biasing the analysis, models were repeated with only calves that were found to harbour apr^R *E. coli* during the sampling period. These repeat tests yielded similar F and *P* values for the effect of age ($F_{1,73} = 0.74$, $P = 0.39$), or sample date ($F_{1,73} = 1.33$, $P = 0.25$) on the presence of apr^R *E. coli*.

Forty-five apr^R *E. coli* were isolated. Genotyping of the isolates by PFGE revealed that the apr^R population was not clonal, and comprised five distinct genotypes (Chapter 4, Figure 4.1a).

The majority (7/9) of samples contained only one genotype (Table 5.1). Two calves (690 and 687) were found to harbour apr^R *E. coli* on more than one occasion. Calf 690 carried different genotypes on the two dates where apr^R *E. coli* were detected, whereas calf 687 harboured the same genotype on all dates of detection. PFGE type

A was the most common genotype and was found in four of the calves. Genotypes C, D and E were only found on one sampling date each. To determine if genotypes were associated with specific sampling dates, the prevalence of each genotype was plotted against the sampling date (Figure 5.1).

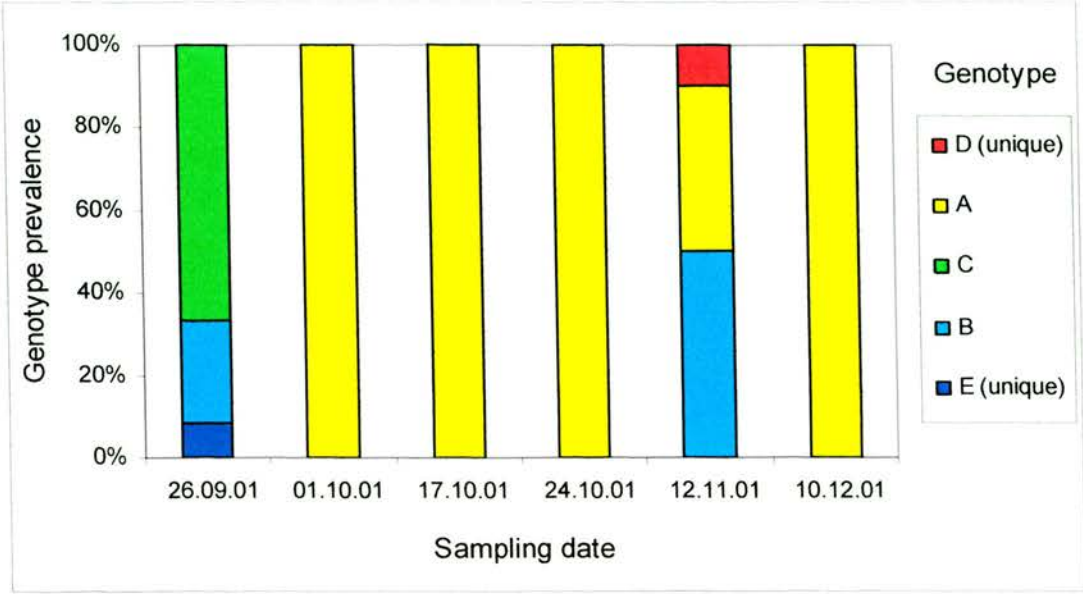


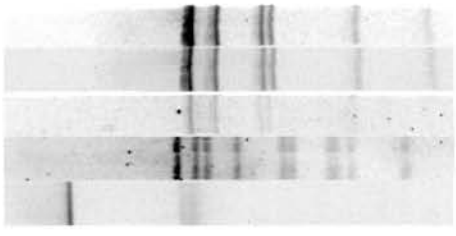
Figure 5.1 Dates of detection and prevalence's of the five different apr^R *E. coli* genotypes.

On four of the six dates on which apr^R *E. coli* were detected, the only genotype present was PFGE type A. All isolates of this genotype (n = 27) were found to have the same resistance phenotype (resistant to tetracycline, sulphamethoxazole, apramycin, tobramycin and gentamicin). On two sampling dates (26/09/01 and 12/11/01) more genotypic diversity (three different genotypes) was present. These are the only two dates on which apr^R *E. coli* were isolated from more than one calf.

5.3b Plasmid analysis

Plasmid profiling with *Eco*RI revealed three different plasmids, designated pUK2001, pUK2002, and pUK2003, of size 91Kb, 115Kb and 181Kb respectively.

The *Eco*RI restriction profiles of these plasmids are shown in Figure 5.2. Plasmid types were confirmed with *Apa*I restriction (results not shown). pUK2002 and pUK2003 were found in only one genotype each, but pUK2001 was found in three different genotypes indicating horizontal spread. It was not possible to restrict plasmid pUK2003 despite repeated attempts, but the different sizes and transfer frequencies (described below) indicate that pUK2003 and pUK2002 are different plasmids.



Plasmid restriction	Calf	Plasmid	Host genotype
	LEO1.0690	pUK2001	A
	LEO1.0693	pUK2001	Unique
	LEO1.0694	pUK2001	C
	LEO1.0696	pUK2002	Unique
	LEO1.0690	pUK2003	B

Figure 5.2 *Eco*RI plasmid types and the host genotypes from which they were isolated.

5.3c Transfer frequencies

All three apramycin resistance plasmids were conjugative. Transfer frequencies were measured between the *E. coli* K12 derivatives J62-2 and J53, using the different auxotrophisms of these strains as a selective mechanism. The transfer frequencies of each plasmid, and the selective plate used for transconjugant enumeration are listed in Table 5.2.

Table 5.2 Plasmid transfer frequencies of pUK2001, pUK2002 and pUK2003, measured on different antibiotic containing selective plates.

Plasmid	Selective plate	Transfer frequency
pUK2001	16mg/L apramycin	4.19×10^{-2}
pUK2002	16mg/L apramycin	1.73×10^{-3}
	10mg/L tetracycline	1.57×10^{-3}
	16mg/L apramycin + 10mg/L tetracycline	1.37×10^{-3}
	8mg/L streptomycin	1.87×10^{-3}
	16mg/L apramycin + 8mg/L streptomycin	2.02×10^{-3}
pUK2003	16mg/L apramycin	4.64×10^{-6}
	10mg/L tetracycline	4.27×10^{-6}
	16mg/L apramycin + 10mg/L tetracycline	5.18×10^{-6}
	8mg/L streptomycin	4.41×10^{-6}
	16mg/L apramycin + 8mg/L streptomycin	4.27×10^{-6}
RP4	10mg/L tetracycline	1.0×10^{-4}
R46	10mg/L tetracycline	2.1×10^{-5}

Plasmid pUK2001 transferred at the highest frequency (4.19×10^{-2}), which was substantially greater than that of the standard plasmids RP4 and R46 (Table 5.2). Plasmid pUK2002 also transferred at a frequency faster than the standard plasmids (Table 5.2). The similar transfer frequencies observed when pUK2002 or pUK2003 were selected on tetracycline, streptomycin or these antibiotics in combination with apramycin, indicate that tetracycline and streptomycin resistance determinants are also carried by these two plasmids. This was supported by measuring the MICs of the donor strains, recipient J53, and transconjugants.

Table 5.3 shows the MICs of the recipient strain J53, the original donor strains (WF006555Y17 to WF007225Y01), and the transconjugants. The plasmid harboured by each of the donors is listed in the second column.

Table 5.3 MICs of wild type apr^R *E. coli* donor isolates and the transconjugants formed after the conjugation of the apr^R plasmids into J53.

Isolate / strain	Plasmid	MIC (mg/L)						
		APR	TOB	GNT	STP	AMP	TET	SUL
J53		2	0.25	0.25	2	4	1	8
WF006555 Y17	pUK2001	>128	64	16	2	4	2	8
WF006562 Y14	pUK2001	>128	32	8	2	4	2	8
WF006716 Y17	pUK2001	>128	32	8	16	4	128	>128
WF007204 Y15	pUK2002	>128	16	8	32	4	>128	8
WF007225 Y01	pUK2003	>128	16	8	32	>128	>128	>128
pUK2001 in J53	pUK2001	>128	16	8	2	4	1	8
pUK2002 in J53	pUK2002	>128	16	8	16	2	>128	8
pUK2003 in J53	pUK2003	>128	16	8	16	2	>128	8

APR, apramycin; TOB, tobramycin; GNT, gentamicin; STP, streptomycin; AMP, ampicillin; TET, tetracycline; SUL, sulphamethoxazole.

In addition to apramycin resistance (MIC >128 mg/L) all three plasmids conferred resistance to tobramycin and gentamicin (MICs of 16 and 8 mg/L respectively). Plasmids pUK2002 and pUK2003 also conferred resistance to streptomycin (MIC of 16 mg/L) and tetracycline (MIC >128 mg/L).

The ability of other commensal *E. coli* from the unselected population to accept plasmid pUK2001 was studied in 28 different genotypes. Of these, 14 were able to receive pUK2001. The frequencies at which this plasmid was transferred to these 14 isolates are shown in Figure 5.3.

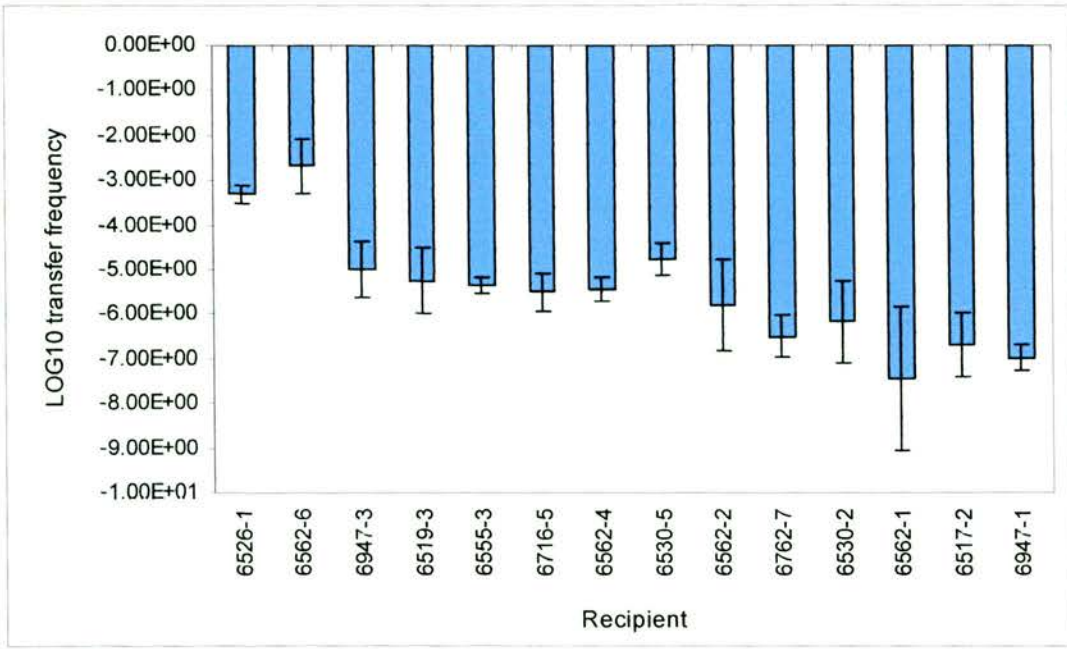


Figure 5.3 Mean transfer frequencies of pUK2001 into 14 different recipients from the unselected *E. coli* commensal population. Error bars show $\pm$ 2x the standard deviation.

The transfer frequencies of pUK2001 into different commensal *E. coli* from the unselected population ranged from 2.03×10^{-3} to 3.43×10^{-8} (Figure 5.3). Fourteen strains were unable to accept the plasmid. Of the 14 strains able to accept pUK2001 *in vitro*, seven were isolated from samples that also contained genotypes carrying pUK2001. Two genotypes (6947-1 and 6947-3) were isolated from a sample from which genotypes presumed to carry pUK2001 were present. This presumption is based on the fact that the presumptive apr^R carriers had identical PFGE patterns to isolates from which pUK2001 was actually extracted, although a plasmid extraction was not carried out on an apr^R isolate from this sample.

5.3d Amplification of the aac(3)IV gene

PCR with primers based on the published *aac(3)IV* sequence (Bräu *et al.*, 1984) yielded a product of the expected size (836bp). A comparison of the sequenced

products with the published *aac(3)IV* gene revealed that all three plasmids harboured this gene. The sequence of each product was identical to that of the published nucleotide sequence.

5.4 Discussion

Although the detection of apr^R bacteria isolated from food-producing animals and humans has been reported previously (Chaslus-Dancla *et al.*, 1989; Hunter *et al.*, 1992; 1993; 1994; Johnson *et al.*, 1994; 1995) these reports have concentrated on organisms that are pathogenic either to humans or animals. Little is known about the epidemiology of resistant commensal bacteria, which constitute a huge reservoir of resistance genes (Schwarz *et al.*, 2001). In this study, PFGE and detailed molecular typing of the resistance mechanism were combined to give a better understanding of the epidemiology of apramycin resistance.

A cohort of calves that had not been treated with any aminoglycosides was selected for this work, to avoid any direct selection of apr^R *E. coli* during the study. During the four months sampling period, six of the 11 calves sampled were found to carry apr^R *E. coli* on one or more sampling dates. The presence of apr^R *E. coli* was found to be unrelated to calf age or sampling date. This is in contrast to the detection of ampicillin resistant *E. coli*, which are significantly more prevalent in new-born and young calves (Hoyle *et al.*, 2004). Similarly, other workers report a higher incidence of antibiotic resistance amongst bacteria isolated from calves than from older animals (Hinton *et al.*, 1985; Linton, 1977a).

The farm, from which the isolates in this study came, had not used apramycin since at least July 2000 (the date from which records of drug usage on the farm were kept). During this time only one calf (not sampled in this study) was treated with Penstrep (see Chapter 2), a combination drug containing penicillin and streptomycin. Although streptomycin resistance is conferred by a different resistance mechanism (enzymatic phosphorylation or adenylation of the antibiotic) to that conferring

apramycin resistance (antibiotic acetylation) (Shaw *et al.*, 1993), plasmids pUK2002 and pUK2003 also carry streptomycin resistance, which may indicate cross-contamination of either strains or antibiotics between calves.

The presence of apr^R plasmids in the calves in this study demonstrates the persistence of apramycin resistance without recent selection by apramycin. A similar result has been reported in *E. coli* from disease outbreaks in pigs (Hunter *et al.*, 1994). Antibiotic usage in cattle is dramatically less than the quantities used in pigs (Veterinary Medicines Directorate, 2001), and so the detection of APR resistance in the commensal flora of calves is of significance.

A total of 45 apr^R *E. coli* were obtained from the six calves. The genotypic diversity amongst these isolates was examined using PFGE, which has previously only been applied to pathogenic apr^R organisms. Amongst the 45 isolates, five distinct genotypes were found. Because APR resistance is commonly found to be mediated by transferable plasmids (Hunter *et al.*, 1992; 1994; Johnson *et al.*, 1994; 1995) (although the putative presence of the *aac(3)IV* gene on the chromosome has been reported by Johnson *et al.*, 1994) genotypic diversity amongst apr^R *E. coli* was expected to be high (compared to a non plasmid-mediated resistance mechanism such as quinolone resistance). Johnson *et al.* (1994) reported different serogroups amongst clinical isolates of apr^R *E. coli*, which is consistent with our findings of genotypic diversity within the apr^R *E. coli* population. Similarly, aminoglycoside resistance amongst different salmonella serotypes (Wray *et al.*, 1986) and genotypically diverse enterococci isolates has been described (Donabedian *et al.*, 2003b; Papaparaskevas *et al.*, 2000). Further PFGE studies on a greater number of isolates would be required to determine the extent to which apramycin resistance plasmids have spread to different *E. coli* genotypes.

The most prevalent apramycin resistant genotype (type A) was detected on five of the six dates where apr^R *E. coli* were recovered. This genotype was also resistant to tetracycline and sulphamethoxazole in addition to the aminoglycosides tested. The high prevalence of this strain in calves that have never been treated with tetracycline,

sulphamethoxazole or aminoglycosides shows the signature of antibiotics used previously or the cross-contamination of resistant strains within the farm.

The sampling dates where more genotypic diversity was detected were those in which apr^R *E. coli* were recovered from different calves. This would suggest that the intestinal flora of each calf differs in the prevalence and range of genotypes present. This hypothesis is supported by Howe *et al.* (1976a; 1976b), where a total of 103 different O-antigen serotypes of commensal *E. coli* were found colonising the intestines of calves. Different selective forces may be exerted on each ecosystem, such as the use of antibiotics, disinfectants, initial colonisation of the gut by different micro-organisms, and the production of bacteriocins by other flora colonising the intestine. Any apr^R *E. coli* present will be subject to these selective pressures and are therefore likely to differ between calves. The study of the predominate genotypes in cattle, and any associations between genotype and animal, would require the genotyping of apr^R *E. coli* from a larger cohort of animals. Work is currently underway to address this issue.

Restriction analysis of the apramycin resistance plasmids from the five apr^R genotypes demonstrated that the gene conferring resistance was carried on diverse plasmids. Similar findings have been reported in pathogenic bacteria, based on incompatibility groups (Wray *et al.*, 1986) and restriction endonuclease fragmentation pattern (Platt & Smith, 1991; Pohl *et al.*, 1993). Sequencing analysis of the *aac(3)IV* gene revealed identical genes in all apr^R plasmids. This degree of plasmid diversity and gene homology suggests that the *aac(3)IV* gene is carried on a transposable element. Previously, the *aac(3)IV* gene has been found to be associated with insertion sequences (Bräu *et al.*, 1984; Salauze *et al.*, 1990) which is compatible with this hypothesis. This would greatly increase the potential for dissemination of the gene to different replicons.

The apr^R plasmids were all conjugative. The frequency at which the transfer of apr^R plasmids occurs is rarely measured, but one study has measured transfer frequencies of apr^R plasmids isolated from *E. coli* and salmonellae isolated from disease

outbreaks of cattle, pigs, sheep and poultry (Wray *et al.*, 1986). Two Apr^R *E. coli* isolates from diseased cattle were found to transfer apramycin resistance at frequencies of $<10^{-8}$ and $<10^{-10}$ transconjugants per donor overnight. Higher transfer frequencies were detected in three apr^R *E. coli* isolated from pigs, where frequencies of 10^{-3} and 10^{-2} per donor were detected when the plasmids were transferred to secondary recipients during a one hour mating (Wray *et al.*, 1986). A seven-hour mating was required to give the similar transfer frequencies detected for plasmids pUK2001 and pUK2002 in this work. In the following chapter, mating times of one hour were studied, which are directly comparable with those described by Wray *et al.* (1986). In addition to these high transfer frequencies, the use of PFGE to genotype each isolate allowed the detection of one plasmid (pUK2001) in three different genotypes. To the best of my knowledge, this is the first report of horizontal spread of apramycin resistance in commensal organisms.

The ability of wild type isolates to accept apr^R plasmids has not been studied previously, and there are very few publications in which the recipient ability of wild-type strains is assessed. Of 28 different genotypes of commensal *E. coli* from the unselected population, 14 were able to accept pUK2001. Of interest is the fact that nine of these recipients were isolated from samples in which isolates containing (or presumed to contain for two isolates) pUK2001 were present. The donor and recipient genotypes are likely to be at similar cell densities within the calves because they were isolated from the same diluted sample. These results indicate that there are barriers to *in vivo* conjugation that may not be present *in vitro*. Resource limitation and conditions of stress have been found to decrease the rate of plasmid transfer (Arana *et al.*, 1997; Curtiss *et al.*, 1969; Muela *et al.*, 1994), and the use of rich media has been shown to increase the probability of mating pair formation compared to minimal media (Curtiss *et al.*, 1969). Some kind of selective pressure may be required in order for the donor bacterium to expend energy and resources in transferring its plasmid. This may have been the use of apramycin many years ago, or resistance to disinfectants or feed supplements, resulting in the spread of pUK2001 into three different genotypes. The calf may have acquired other genotypes capable of accepting the plasmid at a later date, but without any selective pressure

the plasmid may just remain in these three genotypes only. Alternatively, the trigger for transfer may be related to growth phase, and other workers have shown that donor ability is greatest during exponential phase growth (Curtiss *et al.*, 1969). The dynamics of plasmid transfer are discussed in more detail in Chapter 6.

The selective pressures maintaining these large conjugative resistance plasmids in the commensal flora are not clear. The calves sampled were not treated with aminoglycosides or tetracyclines. The presence of pUK2002 and pUK2003 suggests that the spread of plasmids or resistant strains resulted from previous tetracycline or streptomycin use. pUK2001 only carried apramycin resistance. The selective force driving the spread of this plasmid may be previous farm usage of apramycin, or the employment of disinfectants or feed supplements on the farm. Sequencing of the plasmids, which is currently underway, may reveal the presence of such resistance determinants. Unfortunately, it was not possible to determine when in the evolutionary history of the strains acquisition of pUK2001 occurred.

The presence and spread of apr^R plasmids in commensal *E. coli,* under unknown selective pressures, poses important implications for the transmission of this resistance determinant into clinical bacteria.

Chapter 6. The study of plasmid dynamics

6.1 Introduction

In the previous chapter, the transfer frequencies of each apramycin resistance plasmid found in the calf faecal commensal *E. coli* population were defined as the number of transconjugants divided by the number of donors at the start of mating (Provence & Curtiss, 1994). The initial density of the donors and recipients, and the time permitted for mating, were kept constant to allow an accurate comparison of transfer frequencies between the apr^R plasmids, and with the well-characterised plasmids RP4 and R46.

In this chapter, a second method of quantifying plasmid transfers originally described by Simonsen *et al.* (1990), was used. The method, designated the end-point method, is described by the authors as one which "overcomes the shortcomings of previous techniques because it is not affected by cell density, donor: recipient ratio and mating time".

There are three reasons for calculating the plasmid transfer frequencies by two different methods. Firstly, this allows a comparison of the methods to determine the advantages and disadvantages of each. Secondly, different researchers have used a variety of methods to calculate plasmid transfer frequencies, and so the use of two methods allows a comparison of transfer frequencies with a greater number of published results than if only one method was used. Lastly, the results from the end-point calculation may be incorporated into a mathematical model, originally presented by Levin *et al.* (1979). The model describes bacterial cell growth and plasmid transfer in batch culture, and may be used to explore the dynamics of plasmid transfer by altering input variables and observing their effects.

This chapter aims to address the following questions:

1. In what manner do plasmid transfer frequency estimates vary as a function of mating time?
2. What are the advantages and disadvantages of each technique?
3. How do the transfer frequencies/rates measured by each of the methods compare?
4. Does the published model of bacterial cell growth and plasmid transfer fit the experimental data of pUK2002 transfer in batch culture?
5. If the model does not fit, what biological processes are occurring which are not taken into account by the model?
6. Can the model be used to address questions of plasmid persistence in natural populations?

6.2 Materials and Methods

6.2a Bacterial strains and plasmids

The plasmids used in this comparative study were the apr^R plasmids identified in Chapter 5 (pUK2001, pUK2002 and pUK2003). The plasmid pUK2002 was chosen for the more detailed study of variation within and between the two methods of calculating plasmid transfer frequencies. This plasmid was chosen because it had an intermediate transfer frequency in relation to the other apr^R plasmids, and the transfer frequency measured in Chapter 5 indicated that this plasmid was unlikely to be derepressed for transfer. To determine plasmid transfer rates by the end-point method, plasmids were maintained in *E. coli* K12 strain J53 (*pro⁻ met⁻*) (Bachmann, 1972). The *E. coli* K12 strains J62-2 (*lac-, pro⁻ trp⁻ hist⁻*) (Bachmann, 1972) and MG1655kan (Δ *lac*, Km^R) were used as recipients.

6.2b Conjugations

To explore the effect of mating time on the estimates of plasmid transfer frequency, transfer frequencies were determined as described in Chapter 5, but over mating times of 1, 7, 10 and 16 hours. To determine rates of plasmid transfer as estimated by the end-point method, matings were carried out in 50 ml Erlenmeyer flasks containing 10 ml LB (Lennox L broth) (Invitrogen). Overnight cultures of donor and recipient were diluted 1:10 in saline and used to inoculate the flasks, to give an initial density of approximately 1 x 10^6 cfu/ml and a 1:1 ratio of donors to recipients. The cultures were incubated at 37°C with shaking at 150 rpm, and sampled at 30 min intervals after initial inoculation (t = 0) for six hours. Donor, recipient and transconjugant cell densities were estimated by serial dilution in saline and selective plating. In matings between J53 and J62-2, donors were selected on Davis Mingioli (DM) plates supplemented with 50mg/L proline and 50mg/L methionine. Recipients were selected on DM plates with 50mg/L proline, 50mg/L tryptophan and 50mg/L histadine, and transconjugants selected on MacConkey plates containing 16mg/L apramycin and 20mg/L rifampicin. When plasmids were transferred between J53 and MG1655kan, donors and recipients were distinguished by colony colour on the same plate. Here S-gal plates (Sigma) were used, which contain a chromogenic ß-galactosidase substrate. Cleavage of this substrate results in the black coloration of colonies. Recipient cells MG1655kan have a deletion of the *lacZ* gene, resulting in the presence of white colonies. Transconjugants were selected on S-gal plates containing 40mg/L apramycin and 32mg/L kanamycin. Growth rate estimates were based on the average of three replicates. The exponential phase population growth rate was assumed to be the average of the individual growth rates of the donor and recipient, again based on three replicate experiments.

6.2c Estimates of plasmid transfer frequencies and rates

Plasmid transfer rates were estimated as a function of the cell densities of donors (*D*), recipients (*R*) and transconjugants (*T*), during exponential growth, in accordance with the following equation (Simonsen *et al.*, 1990):

Equation 1.

$$\gamma = \psi\left(\frac{1}{N - N_0}\right) \cdot \ln\left(1 + \frac{T}{R} \cdot \frac{N}{D}\right)$$

Where ψ is the average growth rate per hour of the two strains (*D* and *R*) involved in the mating during exponential growth, *N*, the total cell density at the time of sampling, and N_0, the total cell density at the start of mating.

Plasmid transfer frequencies were also determined as the number of transconjugants to donors at the start of mating, as described in chapter 5. Although this method is often quoted as the T/D ratio, mathematically this is not strictly a ratio because T and D are measured at different time points during the experiment (T is measured at the end of the mating and D, at the start). To make clear this distinction, the transfer frequency as estimated by this method will be referred to as T/D_0.

6.2d Model theory

The model used to explore this system was that presented by Levin *et al.* (1979) to describe cell growth and plasmid transfer in a batch mating culture. Here, changes in the densities of donors, recipients and transconjugants due to cell growth and plasmid transfer are considered with respect to time, according to the following equations:

Equation 2.

$$\frac{dD}{dt} = \psi(C) \cdot D$$

Equation 3.

$$\frac{dR}{dt} = \psi(C) \cdot R - \gamma(C) \cdot R \cdot (D + T)$$

Equation 4.

$$\frac{dT}{dt} = \psi(C) \cdot T + \gamma(C) \cdot R \cdot (D + T)$$

Equation 5.

$$\frac{dC}{dt} = -\psi(C) \cdot (R + D + T) \cdot e$$

Where D, R and T are the densities (cfu/ml) of the donors, recipients and transconjugants respectively. ψ(C) is the growth rate per hour, ψ, at a given concentration, C, of resource (mg/L), and e (μg) the amount of resource required to make a new cell. The exact concentration of resource in 10ml LB could not be calculated, so an estimated concentration of 500 μg/mL was used in the model, based on trial and error against experimental results. The plasmid transfer rate constant, γ, (ml per cell h^{-1}) is calculated as described above. Growth rates and plasmid transfer rates are assumed to be Monod functions (Monod, 1949) of resource concentration (C). Growth and conjugation cease when resources are exhausted, according to the following equations:

Equation 6.

$$\psi(C) = \psi_{\max} \cdot \frac{C}{(Q + C)}$$

Equation 7.

$$\gamma(C) = \gamma_{\max} \cdot \frac{C}{(Q + C)}$$

Here, ψ_{max} and γ_{max} are the maximum growth and plasmid transfer rates respectively, and Q, the half saturation constant (mg L^{-1}).

There are four basic assumptions of the model, which are summarised as follows:

1. Mating occurs randomly with a frequency that is jointly proportional to the donor and recipient densities, and independent of bacterial growth rates.
2. Plasmids are not lost by segregation.
3. Transconjugants are immediately able to transfer the plasmid at the same rate as donor cells.
4. All clones grow at the same rate.

Model simulations were produced with Berkley Madonna™ differential equation solving package (www.berkleymadonna.com).

6.2e Growth curves

Growth curves were measured in 50 ml Erlenmeyer flasks containing 10 ml LB (Lennox L broth) (Invitrogen). Overnight cultures of plasmid-harbouring J53 (J53pUK2002) were diluted 1:10 in saline and used to inoculate the flasks, to give an initial density of approximately 1 x 10^6 cfu/ml. Cultures were incubated at 37°C with shaking at 150 rpm, and sampled at 30 min intervals since initial inoculation (t = 0) for 5.5 hours. Cell densities were estimated by serial dilution in saline and selective plating onto MacConkey plates supplemented with 16mg/L apramycin.

6.2f Statistics

To analyse the variation in plasmid transfer frequencies as a function of time and plasmid, analysis of variance and multiple linear regressions were performed. In all tests, the number of degrees of freedom is written as a subscript of the test statistic and a significant result was recorded if $P < 0.05$. Linear mixed effects models were used to test whether log-transformed transfer rates differed significantly with mating time. As the temporal data were non-independent, each replicate was entered as a random effect.

6.3 Results

6.3a Effect of mating time on estimates of plasmid transfer frequency

Mean transfer frequencies, determined as T/D_0, were calculated from replicate matings of 1, 7, 10 and 16 hours. Transfer frequencies of pUK2003 could not be determined within a one-hour mating period, as this plasmid required longer than one hour for its transfer. The transfer frequencies of each of the plasmids during the four different mating times are shown in Figure 6.1. Table 6.1 shows the mean transfer frequency and number of replicates used in the estimates.

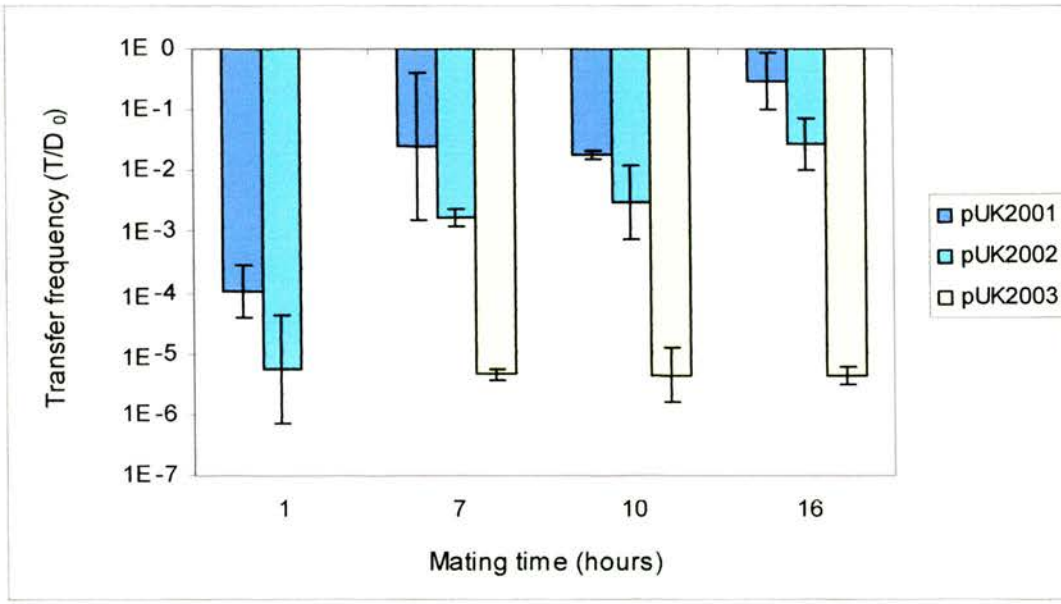


Figure 6.1 The effect of mating time on estimates of plasmid transfer frequency as determined by the number of transconjugants to donors at the start of mating.

Table 6.1 Mean transfer frequencies (T/D_0) and number of replicates used in estimates.

Plasmid	Mating time (no. of replicates) 1	7	10	16
pUK2001	1.14×10^{-4} (8)	4.19×10^{-2} (3)	1.84×10^{-2} (2)	3.07×10^{-1} (2)
pUK2002	9.46×10^{-6} (35)	1.73×10^{-3} (5)	3.41×10^{-3} (5)	3.03×10^{-2} (3)
pUK2003	ND	4.65×10^{-6} (5)	4.84×10^{-6} (5)	4.43×10^{-6} (3)

As can be seen in Figure 6.1, the estimate of plasmid transfer frequency varies according to the time allowed for mating. Transfer frequencies of pUK2003 appeared least affected by mating time, whilst pUK2002 and pUK2001 showed general trends towards increased frequencies during longer mating times. The analysis of variance performed on this data confirmed these trends; a significant increase in transfer frequencies was present with increasing length of mating time ($F_{1,70} = 255.3$, $P < 0.001$). The plasmid transfer frequencies were significantly different ($F_{2,70} = 214.6$, $P < 0.001$), and the relationship between time and transfer frequency was found to be plasmid dependant ($F_{2,70} = 25.47$, $P < 0.001$), with pUK2001 and pUK2002 transfer frequency estimates increasing with mating time ($t_{13} = 9.9$, $P < 0.001$; and $t_{46} = 17.85$, $P < 0.001$ respectively). pUK2003 transfer frequencies did not change significantly with mating time ($t_{11} = -0.23$, $P = 0.82$) (Figure 6.1).

6.3b Transfer rates estimated by the end-point method

The end-point method has been described as being independent of mating time because transfer rates and growth rates during exponential growth are assumed to be related in accordance with equation 1 (Simonsen *et al.*, 1990). To determine when during the course of the experiment, the exponential growth phase is reached, growth curves of the donor strain (J53) harbouring pUK2002 were plotted. The growth curves were determined in eight replicate experiments carried out in the same type and volume of broth, and under the same experimental conditions, as would be used

for determining plasmid transfer rates. The individual growth curves of the replicate experiments are shown in Figures 6.2, and the geometric mean growth curve of all replicates is shown in Figure 6.3.

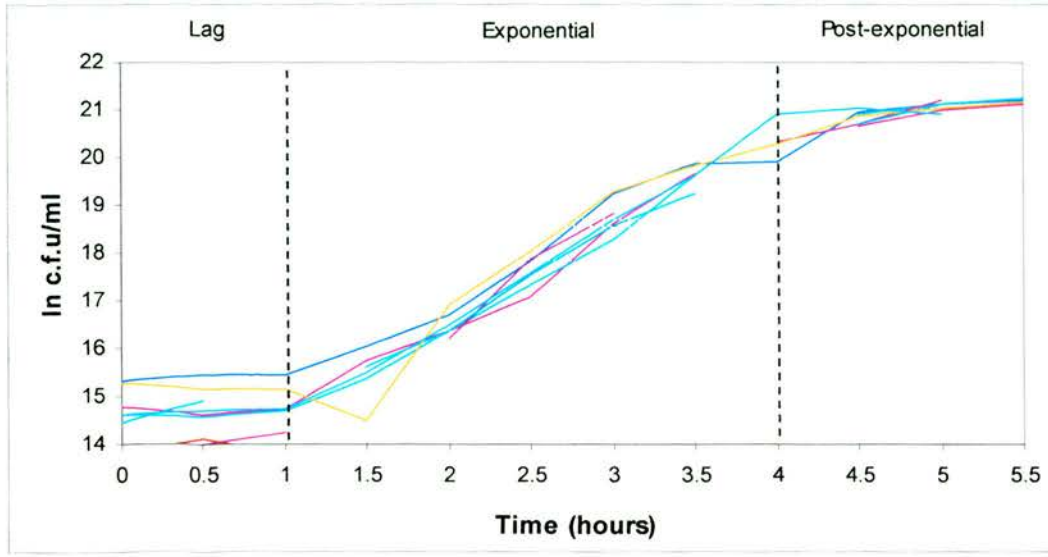


Figure 6.2 Eight replicate growth curves of J53 harbouring plasmid pUK2002 in 10ml LB.

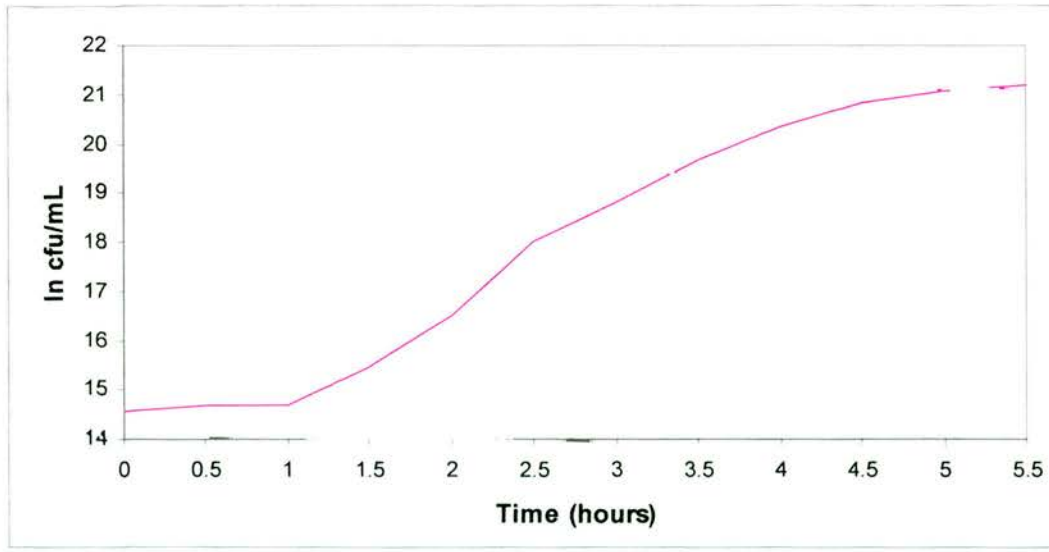


Figure 6.3. Geometric mean growth curve of J53 harbouring pUK2002 10ml of LB.

Figure 6.2 shows that a lag phase occurs during approximately the first hour of incubation. This is followed by an approximately exponential growth phase spanning from one to approximately four hours, after which the growth rate of this strain declines. Figure 6.3 shows the geometric mean of these growth curves. In this graph, the start of the exponential phase is distinct, but the end of this phase is less well defined. To determine if plasmid transfer rates as measured by the end-point method were independent of time of sampling, transfer rates of pUK2002 were determined on the proportion of donors, recipients and transconjugants at each time point. For each transfer rate estimate, the growth rate specific to that time point was used. Here, average growth rates for each of the time points were calculated by performing linear regression of the slope between pairs of consecutive time points for each replicate growth curve, and taking the mean of these rates. Transfer rates at 0-0.5 and 0.5-1 hours were excluded from the analysis because although the mean growth rates of the eight replicate growth curves were very low, some replicates were not growing. Additionally, in two of four replicate mating experiments, the transconjugant population was below the detection threshold of the method (< 100 cfu/mL).

The results of this analysis are presented in Table 6.2, where each transfer rate (with the exception of t = 3.5 and t = 4, where the number of replicates ≤ 2) has been calculated from the geometric means of the cell densities (*D*, *R* and *T*) of replicate experiments. Figure 6.4 shows the transfer rate estimates of all replicates. The numerical values at each time point are listed in Table 6.2.

Table 6.2 Mean transfer rates and confidence intervals for the transfer of pUK2002 from J53 into J62-2 measured after different mating times by the end-point method.

Time (Hours)	No. of replicates	Transfer rate (mL per cell h^{-1})				Mean transfer rate (mL per cell h^{-1})
		Replicate 1	Replicate 2	Replicate 3	Replicate 4	
1.5	3	2.91×10^{-11}	3.23×10^{-11}	9.42×10^{-11}	ND	5.18×10^{-11}
2	4	1.32×10^{-10}	5.36×10^{-11}	8.70×10^{-11}	1.61×10^{-10}	1.08×10^{-10}
2.5	4	5.78×10^{-11}	2.32×10^{-11}	4.88×10^{-11}	2.00×10^{-11}	3.74×10^{-11}
3	4	1.51×10^{-11}	5.21×10^{-12}	7.88×10^{-12}	6.80×10^{-12}	8.75×10^{-12}
3.5	1	ND	2.10×10^{-12}	ND	ND	2.10×10^{-12}
4	2	ND	9.24×10^{-13}	ND	1.22×10^{-12}	1.07×10^{-12}
4.5	3	ND	1.71×10^{-13}	1.42×10^{-12}	1.07×10^{-12}	8.88×10^{-13}
5	3	ND	4.73×10^{-14}	6.00×10^{-13}	3.58×10^{-13}	3.35×10^{-13}

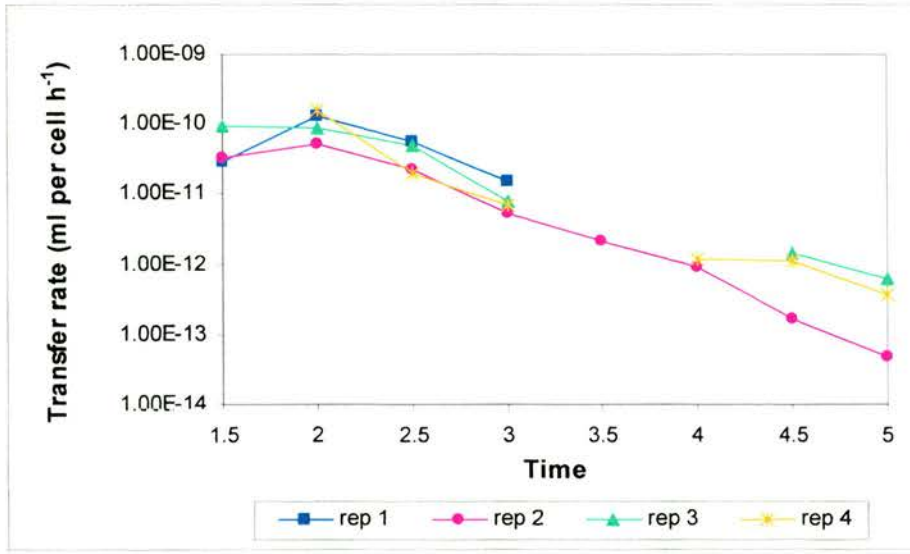


Figure 6.4 Four replicate experiments showing the effect of mating time on estimates of plasmid transfer rate of pUK2002 from J53 into J62-2, as measured by the end-point method.

As can be seen in Figure 6.4, estimates of transfer rate tend to be lower when calculated from cell densities of samples taken later in the mating time. The decrease in transfer rate with mating time was significant ($t_{19} = -5.08$, $P < 0.001$).

The transfer rate appeared to be fastest during the lag phase and slowest after the exponential growth phase when the growth rate is also decreasing. To ensure that the plasmid transfer rate estimates for the three apr^R plasmids were as comparable as possible, and would not be affected by variations in the length of lag phase, they were estimated from cell densities of samples taken at approximately the middle of the exponential growth phase. This will be a conservative estimate, as plasmid transfer rates are thought to be fastest during the lag phase (Simonsen *et al.*, 1990). The plasmid transfer frequencies were then calculated from the cell densities of a sample removed after 2.5 hours of mating. Growth rates were calculated as the rate of growth during exponential phase (1-4 hours). These results are listed in Table 6.3.

Table 6.3 Mean plasmid transfer rates as measured by the end-point method, and mean transfer frequencies as measured by T/D_0.

Plasmid	Plasmid transfer rate (ml per cell h^{-1})	Plasmid transfer frequency (T/D_0)
pUK2001	2.83×10^{-9}	4.19×10^{-2}
pUK2002	3.74×10^{-11}	1.73×10^{-3}
pUK2003	1.15×10^{-15}	4.65×10^{-6}

The transfer rates listed in table 6.3 demonstrate that plasmid pUK2001 has the fastest rate of transfer, and pUK2003 has the slowest. Qualitatively, these results correlate well with the transfer frequencies obtained by calculating T/D_0.

In Table 6.3, different *E. coli* K12 recipient strains were used for the end point matings (MG1655kan) compared to the T/D_0 matings (J62-2). This was to minimise pipetting errors when determining D and R densities with the end-point method. To determine whether the choice of recipient strain used in the mating experiment affected the plasmid transfer frequency obtained, pUK2002 was mated with

MG1655kan and with J62-2, and the transfer rates compared. Conjugations were repeated four times with each recipient. Cell densities were estimated from samples removed after 2.5 hours of mating. The growth rates used in each calculation were the average growth rates of each mating pair during exponential growth (1-4 hours). The results of this analysis are shown in Table 6.4.

Table 6.4 Mean transfer rates of pUK2002 into J62-2 and MG1655kan.

Mating pair	Average transfer rate (ml per cell h^{-1})	Lower 95%	Upper 95%
J53 + pUK2002 into J62-2	3.74×10^{-11}	7.62×10^{-12}	6.72×10^{-11}
J53 + pUK2002 into MG1655	3.53×10^{-11}	9.18×10^{-12}	6.14×10^{-11}

The mean transfer frequencies of pUK2002 into J62-2 and MG1655kan were very similar, and the upper and lower 95% confidence intervals indicate similar variation with the two recipients. A standard two sample t-test revealed that the two transfer systems (into J62-2 or into MG1655kan) did not give significantly different estimates of transfer frequency ($t_5 = 2.57$, $P = 0.87$).

6.3c Variation in transfer frequency/rate with different methodology

To explore the variation over a range of mating times, when the plasmid transfer frequency or rate was measured by each of the two different methods, the results of the mating experiment described in Figure 6.4 were plotted with the T/D_0 frequencies of the same data. The data for each of the four replicates is shown in Figure 6.5.

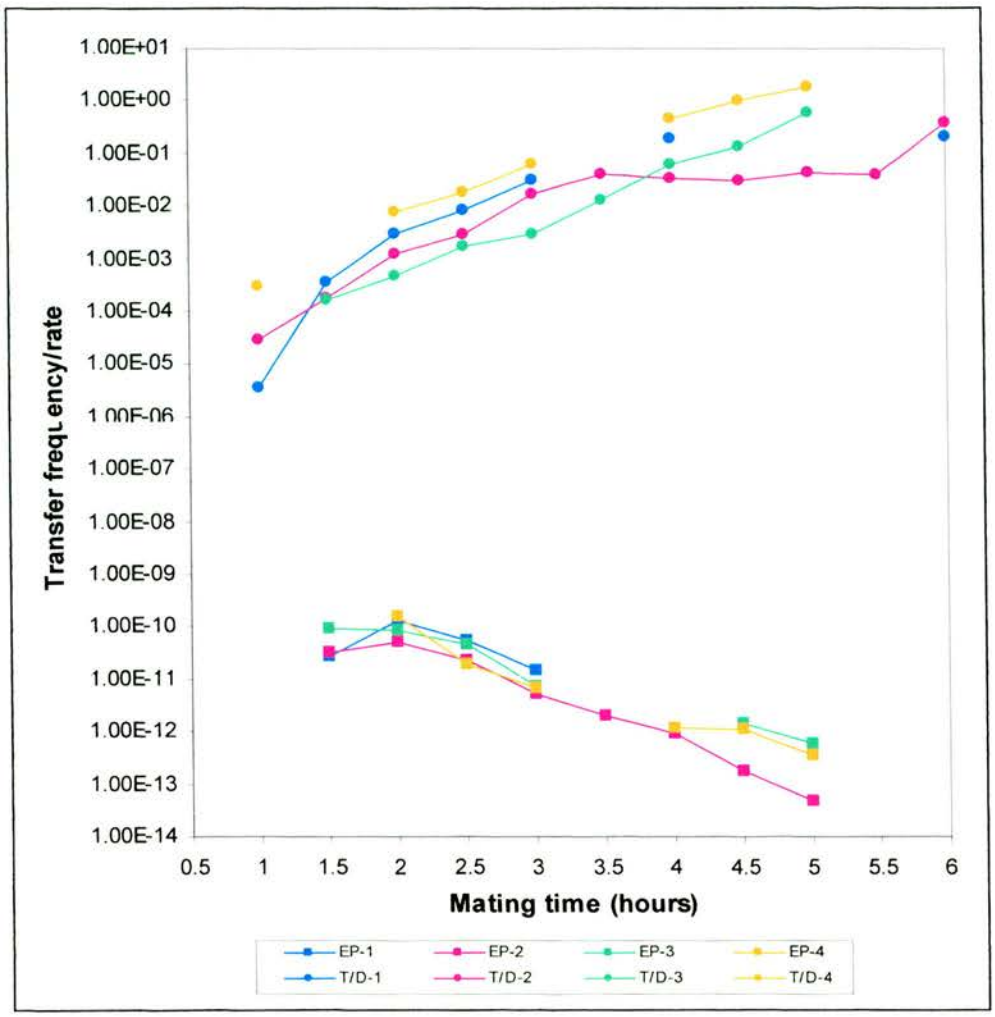


Figure 6.5 A comparison of four replicate experiments to study plasmid transfer. EP1-4; transfer rates measured by the end-point method (mL per cell h^{-1}), T/D 1-4; transfer frequencies measured as the density of transconjugants over donors at the start of mating (T/D_0).

Figure 6.5 shows that neither measure of plasmid transfer is independent of mating time. The frequency of transfer as measured by T/D_0 increases with mating time, whereas the transfer rate, measured by the end-point method, decreases as mating time increases. A significant decrease in transfer rate with mating time ($t_{19} = -5.08$, $P < 0.001$) was described earlier for the end-point method (Figure 6.4). This analysis was repeated for the T/D_0 data, and revealed a significant increase in

transfer frequency with mating time ($t_{33} = 4.82$, $P < 0.001$). The replicate points of the end-point method appear more closely clustered than those of the T/D_0 measure (Figure 5.5), indicating less variation within this method.

6.3d Modelling plasmid transfer in batch culture

A model describing bacterial cell growth and plasmid transfer in batch cultures (see section 6.2d in materials and methods) was used to describe mathematically the experimental results obtained during the calculations of plasmid transfer frequencies by the end-point method, and to predict the effect of altering various parameters where this could not be achieved practically. The plasmid transfer results selected for this section of work were those of plasmid pUK2002. To ensure the model accurately describes the experimental data, the cell densities, plasmid transfer rate, and average growth rates from the mean results of four replicate pUK2002 conjugation experiments were incorporated into the model. The model was then run using Berkley-Madonna™ software and the output compared to the experimental results.

When the model was run using the maximal cell growth rates for each population it did not represent the experimental data. The T, D and R populations were overestimated throughout the five hours mating time (results not shown). The model predicted resource limitation at 2.5 hours, but this was not observed in the experimental data. The results shown in Figure 6.2 demonstrate that growth rates vary during the experiment, and so the model was repeated with the average growth rates over the five-hour experiment for each population. This gave a better representation of the experimental data than when the maximal growth rates were used, but the transconjugant population was still greatly overestimated by the model (results not shown). Table 6.2 and Figure 6.4 indicate that the plasmid transfer rate varies during different growth phases. It was hypothesised that the model would fit the real data better if the average transfer rate during the five-hour experiment was used. This hypothesis was tested and the comparison of real and model data is presented in Figure 6.6.

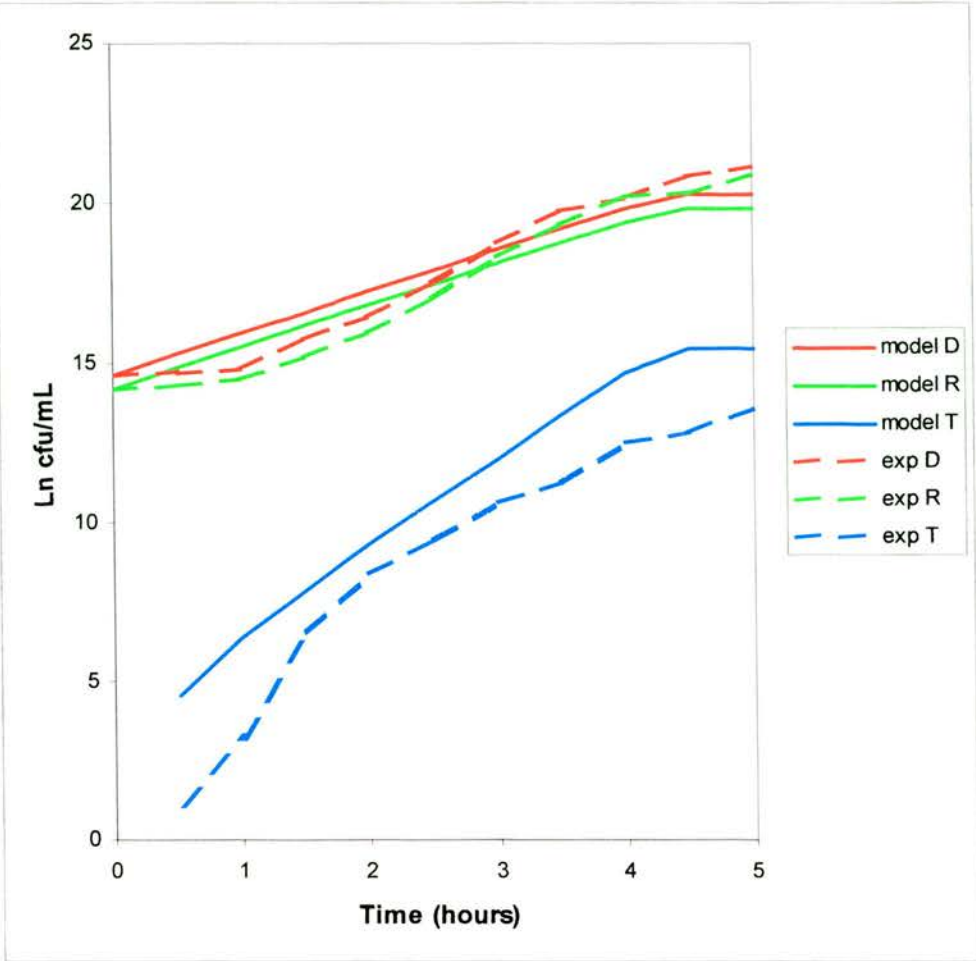


Figure 6.6 Population dynamics during pUK2002 transfer from J53 to J62-2, using the average plasmid transfer frequency over each of the time points.

Figure 6.6 shows that the model-generated data follows the general trend of each of the populations, but it does not capture the sigmoid shape of the D and R experimental curves. Although the slope of the modelled T population is similar to that of the real data, the main discrepancy between the model and real data is that it overestimates the T population throughout the five hours. In an attempt to enhance the fit of the model to the real data, two further models were run in which cell densities, growth rates, and plasmid transfer rates from the lag phase and secondly from the post-exponential phase were used. The use of data from these specific growth phases only, resulted in very inaccurate modelling of all populations, even during the particular growth phases from which the data came (results not shown).

The model shown in Figure 6.6 was also repeated with a range of transconjugant growth rates (vT) from 0 to 2.5, to determine whether the overestimation of T density was because of transconjugant growth. The numerical solutions and the resulting graph for all these vT values were almost identical to those for Figure 6.6 (results not shown), indicating that the transconjugant population plotted in Figure 6.6 is a direct result of conjugation events and that transconjugant growth has an undetectable effect on population density.

A sensitivity analysis was performed to determine how each parameter influences the output of the model. The graphs of this analysis may be found in appendix 1. The key findings of this analysis are as follows:

1. Reducing the donor or recipient growth rates (vD and vR respectively) enhanced the fit of the model to the T population, but resulted in very inaccurate modelling of the D and R populations.

2. When vD > vR, donors constituted a greater proportion of the total population at resource limitation. Conversely, when vR > vD, recipients occupied a greater proportion of the broth than D. The reduction in D as a function of increasing vR, was less than the reduction in R due to increasing vD. This is likely to be because D only acts in competition with the other strains present, whereas R is affected by conversion to T, in addition to competition with both D and T.

3. Lowering the initial densities of D or R to 1×10^5 lowered the T curve to fit the experimental T data much better, but this also caused a lowering of the D and R curves, which was a very poor representation of the experimental data for these populations.

4. There was a trade off between an increase in resource concentration increasing the fit of the D and R populations, but resulting in a reduced fit to the T population.

5. The parameter changes that did enhance the fit of the model to the experimental data were the introduction of a lag in conjugation, increased resources, a lag in growth of all D and R, and a reduction in plasmid transfer frequency. A more detailed discussion of these parameter changes now follows.

A lag in plasmid transfer was introduced by offsetting the model-generated results of the transconjugant population from the corresponding time points by 30, 60 and 90 minutes. The results of this analysis are shown in Figure 6.7.

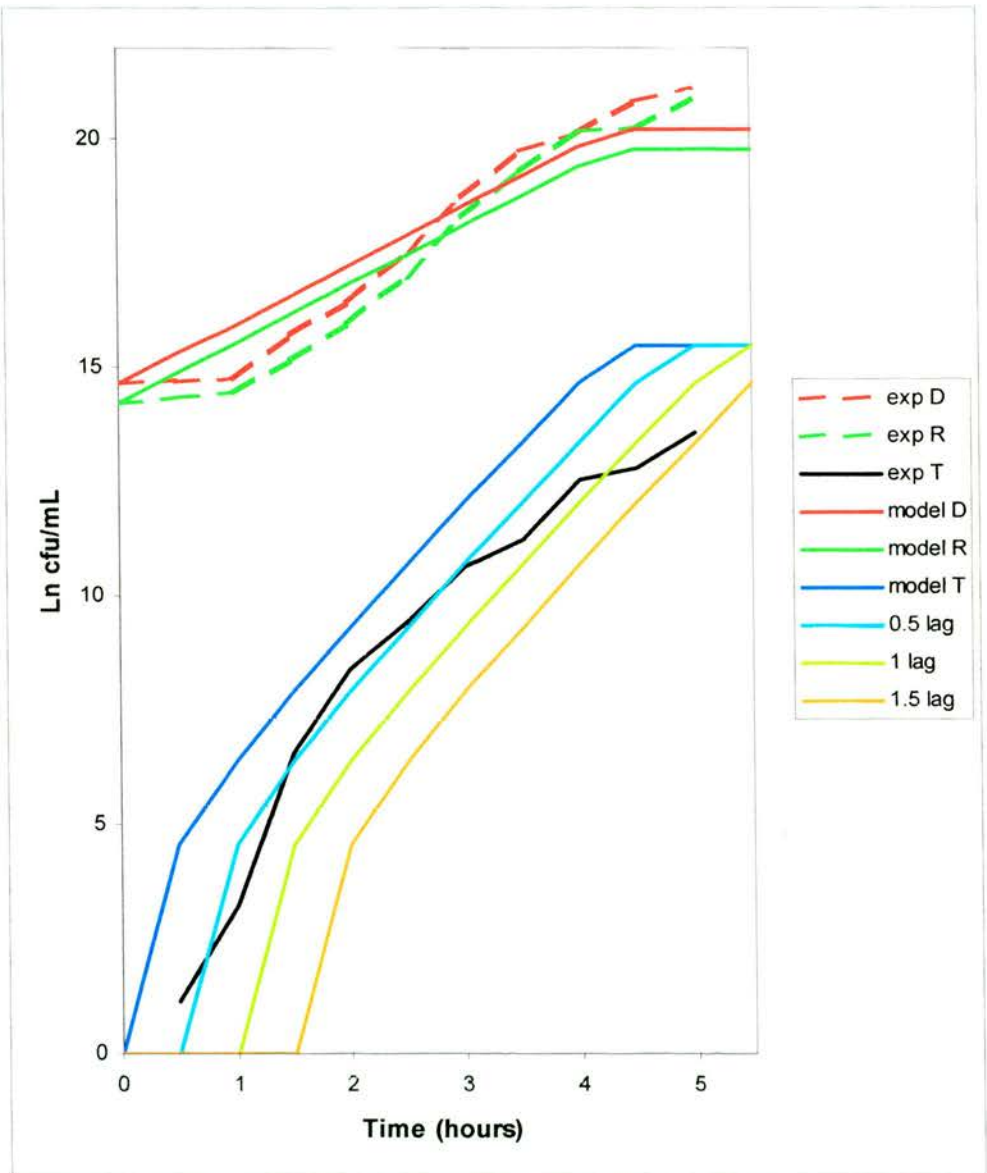


Figure 6.7 The effect of a lag in conjugation on the dynamics of pUK2002 transfer between *E. coli* J53 and J62-2.

The model more accurately represents the experimental data when a 30 minute (0.5 hours) lag in transfer is introduced (Figure 6.7), but after 3 hours the increase in T density is less than is predicted by the model. A lag in growth of approximately one hour also needs to be introduced before D and R growth begins. This is illustrated in Figure 6.8.

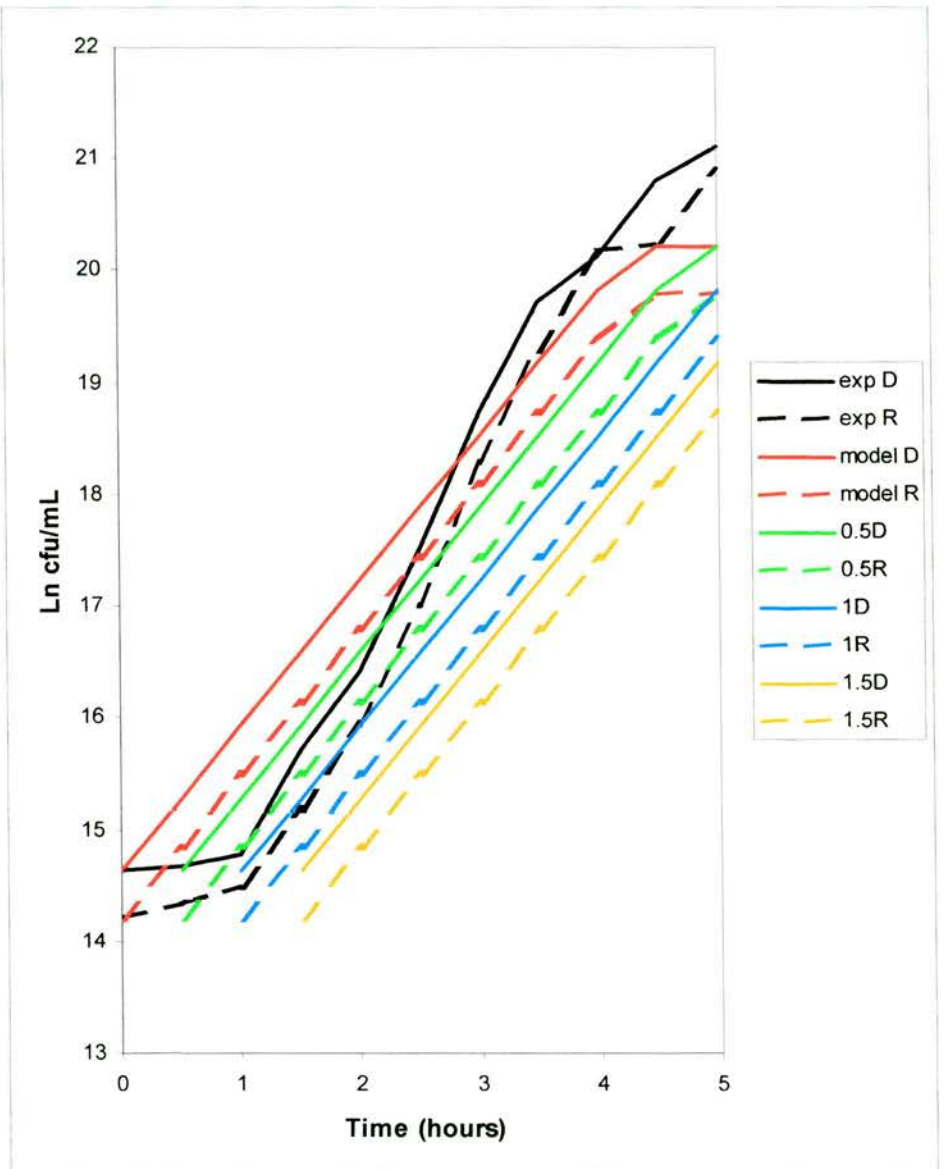


Figure 6.8 The effect of 0.5, 1 and 1.5hrs lag in growth on the D and R populations.

To compensate for the one hour lag before cell growth, the growth rates of the D and R populations needs to be increased. A growth rate of $2.4h^{-1}$ was found to be most suitable.

Next, the effect of varying transfer rate (γ) was explored. This analysis is shown in Figure 6.9, where for simplicity, only the effects on the R and T populations are shown. The D population is only affected by direct competition between the other strains and so is expected to grow according to its growth rate until resource limitation is reached.

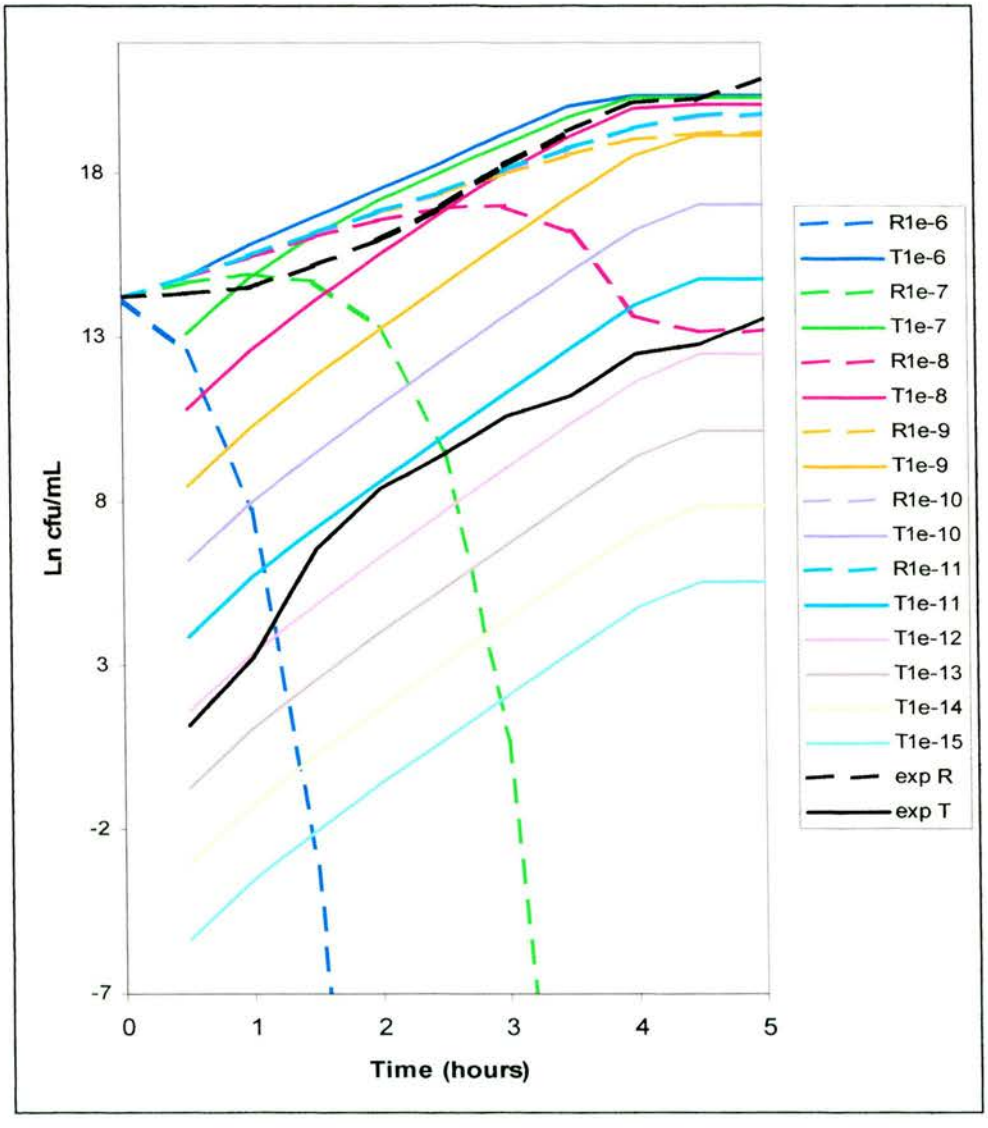


Figure 6.9 The effect of plasmid transfer rate on the R and T populations.

Figure 6.9 shows that a plasmid transfer rate of between $1e^{-10}$ and $1e^{-15}$ does not affect the R population (shown as just a turquoise dashed line; the other colours cannot be seen, as they are directly under this line). At $\gamma \geq 1e^{-9}$, the R population is reduced, and dies out when $\gamma \geq 1e^{-7}$ because all the R's are converted into T's during the course of the experiment. From this figure it appears that the best plasmid transfer rates to fit the experimental data is between 1×10^{-12} mL per cell h^{-1} and 1×10^{-11} mL per cell h^{-1}.

Different combinations of the three parameters found to enhance the fit of the model (lag in transfer, lag in D and R growth, and increased resource concentration) were studied in combination. The results of this analysis may be found in Appendix 2, in graphs 1 to 27.

Figure 6.10 shows the results of the best fit of the model (selected by eye), which includes a 30min lag before plasmid transfer, a 1.5 hour lag before D and R cell growth, D and R growth rates of $2.4h^{-1}$, a plasmid transfer rate of 1×10^{-11} mL per cell h^{-1} and a resource concentration of 500μg/mL. Here it is important to note that the concentrations of resource input into the model are arbitrary. LB contains both tryptone peptide and yeast extract, both of which may be used as a C source. This meant that it was not possible to calculate exactly how much of each different carbon source was initially present and subsequently used up during the experiment.

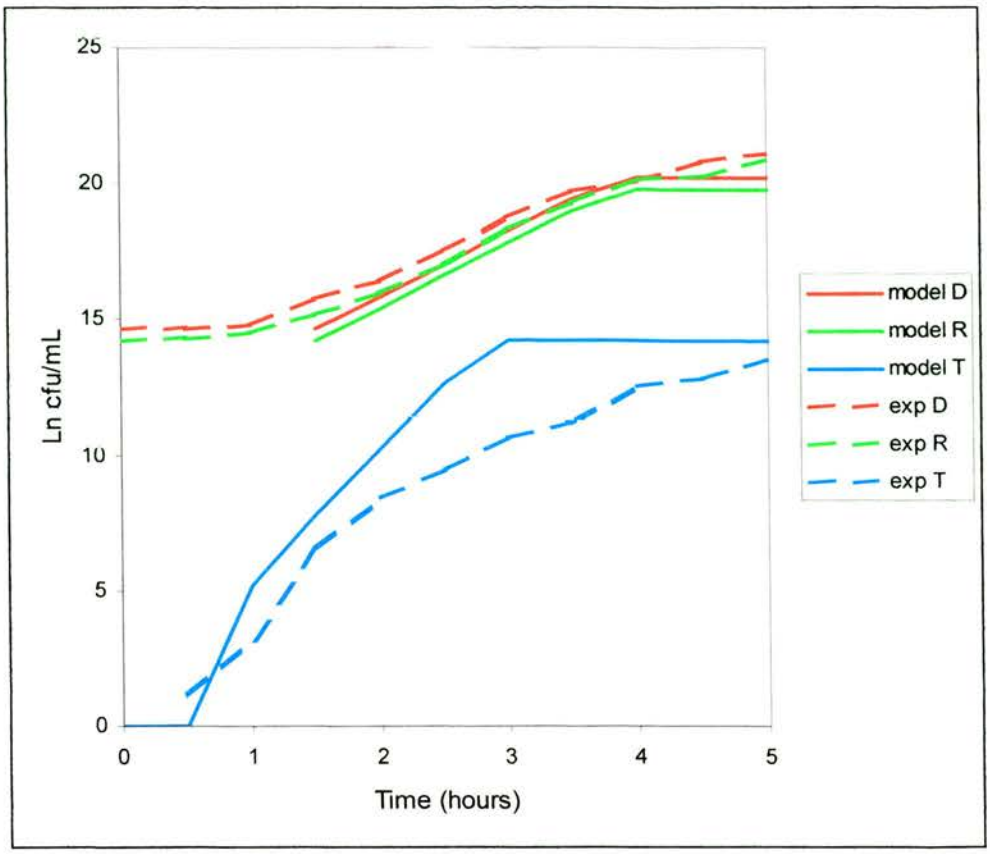


Figure 6.10 Best fit of the model to the experimental data, using measured plasmid transfer rate (1.98×10^{-11} mL per cell h^{-1}).

In Figure 6.10, the density of transconjugants throughout the experiment are overestimated. With a resource concentration of 500μg/mL, the culture reaches resource limitation at 4 hours. Experimentally, resource limitation is not reached until at least 5 hours. However, increasing resources further also affects the T population (see Figure A1 in appendix 1) by increasing the number of transconjugant cells formed. To reduce this, a lower plasmid transfer rate may be used, but this is not logical because the transfer rate is measured during the experiment based on the densities and growth rates of the cells involved. Therefore, either the measurement of transfer rates with the end-point method is not accurate, or the use of the transfer rate in the model requires a further variable to reduce the transfer rate with increasing

mating time. Biologically, the reason for this is likely to be because of a reduction in donor ability during exponential phase growth compared to lag phase.

The final section of this chapter intended to explore which parameters are influential in allowing persistence of the plasmid in a population. The use of an accurate model is critical for this analysis, as it allows the alteration of different parameters in a thorough and stepwise manner, which is not possible experimentally. Although only a crude exploration of parameter space was completed due to time limitations, it is clear from the results above (Figures 6.6 to 6.10) that the model is not able to capture all of the behaviour of the experimental data. The model needs to allow the input of transfer rates that change throughout the experiment, depending on the growth phase of the donor and recipient strains. The application of a mathematical model to the phenomenon of conjugative plasmid transfer has however highlighted processes and dynamics of apr^R plasmid transfer that would not have been observed by simply measuring transfer frequencies or rates.

6.4 Discussion

This chapter is concerned with gaining a better understanding of the biology of the apr^R plasmids described in chapter 5. Two different methods of determining plasmid transfer frequencies were examined to allow a comparison of these two published methods, in addition to a comparison of plasmid transfer frequencies with other worker's results. The use of the end-point method enabled experimental data to be compared with data generated using a mathematical model describing cell growth and plasmid transfer in batch culture. Testing the model against experimental data highlighted limitations of the model and identified biological processes likely to be involved in plasmid transfer. The discussion of the results described earlier will be organised according to the original aims set out in the introduction.

6.4a In what manner do plasmid transfer frequency estimates vary as a function of mating time?

When plasmid transfer frequencies were measured as T/D_0 (where T = cfu/ml of transconjugants, and D_0 = cfu/ml of donors at the start of mating), overall there was a significant increase in transfer frequency estimate with increasing mating time. This is because transconjugants are formed by plasmid transfer to a recipient (horizontal spread) as well as by clonal replication (vertical spread) of a transconjugant cell. The number of transconjugants formed by both modes of plasmid transmission will increase as the mating time is increased. The longer the mating time the greater the effect of transconjugant growth on the number T/D_0, because of the logarithmic nature of bacterial cell growth.

In contrast, the transfer rates measured by the end point method decreased with mating time. The fastest transfer rates occurred during the early exponential growth phase, and a significant decrease in the rate of plasmid transfer occurred during 2.5 to 5 hours of mating. Similarly, Smets *et al.* (1993) found that the highest rates of plasmid transfer were observed when donor cultures were in the exponential growth phase. The decrease in plasmid transfer between 2.5 and 5 hours is because with increasing mating times $Ln(1+T/R \bullet N/D)$ increases, but at a slower rate than the decrease in $1/(N-N_0)$. This indicates that in order to compare results between different labs, the same growth phase should be used in the estimate of transfer rate. This result is in contrast to the results of Simonsen *et al.* (1990) where constant transfer rates were found throughout the exponential, post-exponential and stationary phases of growth.

The correlation of maximum transfer rates with early exponential growth phase contrasts strongly with the results of other workers, where minimum transfer frequencies (measured as the frequency of T/D_0) were measured during the donor exponential growth phase (Muela *et al.*, 1994).

6.4b What are the advantages and disadvantages of each technique?

The measure T/D_0 does not take into account the growth of the transconjugant population. This, as outlined above, may lead to inaccurate transfer frequency estimates with longer mating times. In the determination of transfer frequencies of the three apr^R plasmids identified in chapter 5, a mating time of one hour would have been less affected by transconjugant growth than the seven hours used. However, the minimum mating time that could be used was seven hours, as pUK2003 transfer could only be detected after this time.

Additionally, transfer frequencies obtained by T/D_0 are likely to vary if different donor: recipient ratios are used. A lower initial density of donors to recipients is likely to have more of an effect than a reduction in the initial density of recipients. This is because the expression of genes required for the transfer of most conjugative plasmids occurs in only a small proportion of the cells that harbour them (Hardy, 1984), and so the rate-limiting step would be donor ability. In Enterobacteriacae, the conjugative ability of F-like R plasmids is only expressed in approximately 0.1% of cells containing the plasmid (Hardy, 1984). Walmsley (1976) describes the limiting step in the formation of mating pairs under normal physiological conditions, as the production of F pili by donor cells. Considering both these observations, one would expect that a reduction in donor numbers would be likely to reduce transfer frequencies more dramatically than a reduction in the number of recipients. To test whether this hypothesis is true for the strains, plasmids and media used in these conjugation experiments would require repeat mating experiments with a variety of donor: recipient ratios.

Fernandez-Astorga *et al.* (1992) report constant transfer frequencies in tryptone soya broth, as measured by T/D_0 when the density of recipient cells is higher than that of donor cells. With ratios of 30:1 (D: R) or higher donor densities, a significant decrease in transfer frequency was detected. A ratio of 1:10 (D: R) was used in the measurements of transfer frequencies by T/D_0 in this and the previous chapter,

therefore any variation in inocula (introduced by pipetting errors) were expected to have a minimal effect of the transfer frequency obtained.

In summary, this method of calculating plasmid transfer frequencies allowed an accurate comparison between the three apr^R plasmids and the two reference plasmids (RP4 and R46). In addition, a comparison of the transfer frequencies obtained following the detection of transconjugants on different selective plates, enabled inferences to be made as to whether other resistance genes were present on the same plasmid or different but mobilisable plasmids. The main disadvantage of the T/D_0 measure is that comparisons with other researchers are not possible if slightly different methodologies have been used (for example, initial donor and recipient inoculums and ratios, different mating times and different culture conditions).

The rate of plasmid transfer was next measured by the end-point method. According to Simonsen *et al.* (1990), plasmid transfer and cell growth are thought to remain proportional to each other and to vary little during exponential growth. The period during which exponential growth occurred for the cells and culture conditions used was determined by plotting growth curves. This is an important first step, as the different phases of bacterial cell growth vary in both their presence and duration (Monod, 1949), depending on the media, strain, amount of aeration, and starting temperature.

Transfer rates could not be determined until after one hour of mating, because some replicates were not growing at all, and two of the four replicates did not yield transconjugants at that time. To give an average transfer rate of these replicates would not be an accurate representation of the data. At this early stage in the experiment, the formation of some transconjugants were expected, but their numbers may have been below the detection threshold of the method (<100 cfu/mL), giving the appearance of an absence of plasmid transfer. According to Simonsen *et al.* (1990), plasmid transfer rates are at their maximum during the lag phase, so we would expect the highest rates to occur during the first hour of mating. In agreement

with this statement, Muela *et al.* (1994) demonstrate high transfer frequencies during donor cell lag phase compared to the exponential growth phase.

To sum up, the end-point method is more complicated and labour intensive than the T/D_0 measure, but is less affected by mating time, and demonstrated less variation in replicate experiments. A disadvantage of the end-point method is that the calculation of transfer rates during lag phase may be inaccurate or impossible because of very low or negative growth rates.

6.4c How do the transfer frequencies/rates measured by each of the methods compare?

Two different recipients, J62-2 and MG1655kan were used in the measurement of transfer frequencies/rates by the two methods. The use of a second recipient in the end-point method was to minimise pipetting errors introduced by plating samples onto different agar plates. Before the transfer frequencies/rates could be compared, it was necessary to ensure that the use of different recipients would not affect the results.

The ability of different *E. coli* strains to receive plasmids is likely to vary if the recipient strain has a different restriction modification system to that of the donor cell (Murray, 2002). Gordon (1992) found the variation in transfer rate between *E. coli* isolated from a variety of natural populations to be mainly due to recipient effects. Both the genetic relatedness of the two strains involved, and the native plasmid burden of the isolate influenced its ability to receive plasmid R1 (Gordon, 1992). Mutations affecting the inner core of lipopolysaccharide (LPS) and the porin OmpA have also been found to result in poor recipient ability in broth matings, and are thought to reduce mating pair stabilisation (Klimke & Frost, 1998).

It was expected that MG1655kan would have a very similar if not identical ability to receive plasmids from J53. The main reason for this hypothesis is that it is also a K12

derivative strain, and so is genetically related to both J53 and J62-2. Additionally, MG1655kan does not contain any other plasmids. Transfer rates obtained with the same plasmid and each recipient were not significantly different, and so the frequencies of T/D_0 and the plasmid transfer rates measured by the end-point method could be compared.

Qualitatively, the transfer rates of plasmids pUK2001, pUK2002 and pUK2003 estimated by the end-point method, were found to correlate well with those obtained by measuring T/D_0. Plasmid pUK2001 transferred at the fastest rate (2.83×10^{-9} ml per cell h^{-1}). Plasmids pUK2002 and pUK2003 transferred at slower rates (3.74×10^{-11} and 1.15×10^{-15} ml per cell h^{-1} respectively). A study of the transfer rates of R1 between different *E. coli* strains from natural populations revealed that the fastest rate of transfer for this plasmid was 5.2×10^{-11} ml per cell h^{-1} (Gordon, 1992). Transfer rates varied according to the donor and recipient strains used, and averaged at 1.3×10^{-15} ml per cell h^{-1}. This rate is several log-fold slower than the rate of pUK2001 transfer.

However, the rates estimated in this chapter are likely to be faster than those obtained by mating experiments with the natural host strain. This is because the *E. coli* K12 strains used in this work are well-adapted to culture conditions in the laboratory, and perhaps more importantly, they are isogenic and so there is likely to be no restriction on the transfer of plasmids between them (Murray, 2002). In support of this hypothesis, Gordon (1992) found that the transfer of R1 into the lab strain CSH50 was on average 100 times faster than when wild-type strains served as both donors and recipients.

The transfer rates and frequencies obtained in this and the previous chapter are likely to be the maximum possible for each plasmid. Nutrient rich media was used, the broths were well aerated and constantly mixed, and no other competing bacteria were present. These conditions do not represent the conditions likely to occur in the intestine of a calf. The use of a rich medium has been shown to increase the mean number and length of donor pili and increase the probability of mating pair formation

(Curtiss *et al.*, 1969) compared to minimal media. Starvation prior to mating, or conditions of stress have also been found to inhibit plasmid transfer (Muela *et al.*, 1994; Curtiss *et al.*, 1969; Arana *et al.*, 1997).

6.4d Does the published model of bacterial cell growth and plasmid transfer fit the experimental data of pUK2002 transfer in batch culture?

The transfer rates determined by the end-point method, may be readily incorporated into a mathematical model (Levin *et al.*, 1979) describing bacterial cell growth and plasmid transfer in batch culture. The reason for wishing to use a model in this work was to enable an exploration of the dynamics of plasmid transfer by altering input variables which were not achievable experimentally, and subsequently observing their effects. The model was found to fit the growth curves of the donor and recipient population much better than it did the transconjugant population, although it did not capture all of the behaviour of the data. The rate of plasmid transfer was found to vary depending on the growth phase, a result that has been reported by others (Smets *et al.*, 1993; Muela *et al.*, 1994; Curtiss *et al.*, 1969) as well as demonstrated in this work. Although repeat runs of the model with the average transfer rate over the whole experiment fitted the data better, it was still overestimating the number of transconjugants throughout the mating time. The introduction of a 30 minute lag before plasmid transfer did appear to improve the fit to the T population. This illustrates that the dynamics of transfer of plasmid pUK2002 between J53 and J62-2 are not as simple as predicted by the model, and highlights the need to identify biological processes occurring during the transfer of pUK2002 that are not considered by the model.

6.4e If the model does not fit, what biological processes are occurring which are not taken into account by the model?

Firstly, a lag in growth was detected (Figure 6.2) at the start of the experiment despite the fact that the LB broth was warmed to 37°C prior to inoculation. The presence of a lag phase in bacterial growth has been found to depend on the specific conditions and the properties of the organism (Monod, 1949). Additionally, an apparent lag may be caused if the inoculate contains a large proportion of non-viable cells (Monod, 1949). In the end-point mating experiments described in this chapter, an overnight culture was diluted in total 1 in 1000 into the LB. Although it is not clear what proportion of the total cells present in this inoculum were viable, all of the viable cells would be in stationary phase (after 16-18hrs incubation in 5mls LB) at the moment of inoculation. Here, there will be a delay in growth (the duration of which will depend on the bacteria and the media) while the necessary genes for growth are transcribed and translated into protein products. The model requires a function to allow for this lag in growth, the duration of which *in vivo* is unknown.

Secondly, plasmid transfer has been found to be more frequent during the lag and early stationary phase of donor cells than during the exponential phase (Muela *et al.*, 1994). The results of this chapter suggest that the plasmid transfer rate varies as a function of growth phase, not necessarily growth rate. The model does not take into account any variation in ability to donate or receive plasmids by the strains involved, which has also been found to vary with growth phase (Smets *et al.*, 1993; Fernandez-Astorga *et al.*, 1992).

6.4f Can the model be used to address questions of plasmid persistence in natural populations?

Models simplify observed phenomenon into mathematical terms to determine whether the data can be explained in terms of apparently few parameters and variables, and by doing so have to make assumptions. By studying the assumptions

made, one can determine whether the model is likely to be a realistic representation of plasmid transfer *in vivo*.

The model assumes that the frequency of matings increases at a rate proportional to the increase in donor and recipient populations. A consequence of this assumption is that the mating culture must be subjected to continuous mixing, which increases the likelihood that donor cells will encounter recipient cells. *In vivo* studies have indicated that the majority of plasmid transfers occur in the mucus layer of the intestine (Poulsen *et al.*, 1994). Here the bacteria are thought to retain fixed spatial positions despite peristalsis and the fluidity of the mucus (Licht *et al.*, 1999). These conditions permit little mixing of the population, which is thought to account for the observation of a brief period of rapid conjugation after inoculation in mouse colonisation experiments, followed by no further plasmid transfer (Licht *et al.*, 1999). Additionally, the density of cells able to receive the plasmid is likely to be far lower *in vivo* than in these broth matings, because of differences in restriction modification systems present in other bacteria and even within different *E. coli* strains (Murray, 2002).

A second assumption of the model is that mating frequency is independent of bacterial growth phase. The results described in this chapter demonstrate that the rate of plasmid transfer is affected by the growth phase of the donor and recipient population, because significantly different transfer rates were measured at different mating times, and different growth rates were demonstrated at each of these mating times. Here the fastest rate of transfer occurred during exponential growth of the donors and recipients, and the slowest rates were measured in the lag and post-exponential growth phases. Although other workers have found different relationships between growth rates and plasmid transfer frequencies (Smets *et al.*, 1993; Muela *et al.*, 1994; Curtiss *et al.*, 1969), these studies support the hypothesis that plasmid transfer is not independent of bacterial growth phase.

The model also assumes that transconjugants are immediately able to transfer the plasmid at the same rate as donor cells. Cullum *et al.* (1978) report that most newly

formed transconjugants are only able to mate after approximately 90 minutes. Similarly, Andrup & Anderson (1999) report a "recovery period" of about 40-80 minutes, following acceptance of the F plasmid in *E. coli* or plasmid pCF10 in *Enterococcus faecalis* before tranconjugants mature into proficient donors. These reports indicate that this assumption may introduce inaccuracy into the model.

The are a number of studies which report that the growth rates of plasmid carrying cells are slower than those of plasmid free cells (Gillespie, 2001; Goodwin & Slater, 1979; Helling *et al.*, 1981; Inselberg, 1978; Kayama & Yara, 1975). In contrast, other workers have identified plasmids for which carriage does not impart a fitness cost on the host bacterium (Bouma & Lenski, 1988; Lenski *et al.*, 1994; Enne *et al.*, 2004b). The model used in this chapter assumes that the growth rate of all clones is identical. This is tested in the next chapter, where plasmid-carrying and plasmid-free isogenic strains are grown in competition with each other.

The growth rates of all strains involved is likely to be much lower *in vivo* than in a nutrient rich broth like LB. Generation times of 20mins have been observed in broth cultures compared to a mean generation time of 12h in the alimentary canal (Hardy, 1984). Cell growth and plasmid transfer are energy requiring processes, and so are both likely to be slower when nutrients are more limited than in LB. A comparative study on the transfer rates of the derepressed plasmid R1*drd*19 in batch culture demonstrated transfer rates in mouse caecal contents or mouse caecal mucus were 4- and 50-fold lower than those obtained in L-broth (Licht *et al.*, 1999). *In vivo*, plasmid transfer was only detected during a brief period following inoculation, after which no further transfer occurred, even though rapid division of donors and recipients was observed (Licht *et al.*, 1999). Similarly, Thomas *et al.* (2001) report a reduced plasmid transfer frequency between *Bacillus* spp. in mosquito larvae compared to broth culture. These results support the hypothesis that the transfer rates obtained in the broth mating experiments described in this chapter, are likely to be over-estimates of the rates achievable *in vivo*.

Finally, the model predicts that plasmids with faster transfer rates will transfer to a greater number of recipients, resulting in a greater prevalence than plasmids with lower transfer rates. Of the three apr^R plasmids identified in the calf faecal *E. coli* population, pUK2003 transferred at the slowest rate (Chapter 5), yet it is the second most prevalent plasmid. This demonstrates that the factors responsible for plasmid persistence *in vivo* are more complicated than can be explained simply in terms of growth rates and plasmid transfer rates of the two strains involved in a mating. Other factors that may influence plasmid persistence include the presence of other genes on the plasmids (e.g. bacterial attachment pili, metabolic genes), which may not be expressed *in vitro*, as yet unknown selective pressures (e.g. bacteriocin production by other gut flora), and costs associated with high transmission rates that may be undetectable in the closed *in vitro* system used. More information about the immediate environment in which these apr^R plasmids reside is required before questions about plasmid persistence *in vivo* can begin to be addressed.

In summary, two methods were used to measure the transfer rate or frequency of the apr^R plasmids described in Chapter 5. The measurement of T/D_0 allowed an accurate comparison of transfer frequencies within a study, but was more affected by mating time and showed a greater degree of variation than the end-point method. The end-point method is likely to be more comparable with other workers results because it takes into account the densities of all cells involved in a mating, but problems may arise when attempting to measure transfer rates during lag phase.

Secondly, a model was applied to the experimental data. It proved difficult to get a very close fit of the model with the experimental data, but exploring the effects of altering the parameters enabled the identification of biological processes occurring during the transfer of the apr^R plasmid pUK2002 that may not otherwise have been questioned. For example, the presence of a lag phase prior to conjugation, and the non-linear (sigmoid) shape of transconjugant density demonstrating the different rates of plasmid transfer with growth phase. The study of the underlying assumptions of the model prompts many questions about the process of conjugation both in batch

cultures and *in vivo*. It is this prompting of questions, more than any attempts to use the model in addressing hypotheses on plasmid persistence, which has been useful in the study of the transfer of pUK2002.

Chapter 7. Competitive fitness costs of apr^R plasmids

7.1 Introduction

The three apr^R plasmids identified in chapter 5 were all of high molecular size (>90kb). The carriage of large conjugative resistance plasmids is generally thought to engender a competitive fitness cost on the host bacterium in the absence of selection for the resistance phenotype (Gillespie, 2001; Goodwin & Slater, 1979; Helling *et al.*, 1981). Lower growth rates of plasmid-containing cells compared with plasmid-free cells have been observed in both closed-culture experiments (Inselburg, 1978; Kayama & Yara, 1975) and chemostat systems (Goodwin & Slater, 1979; Noack *et al.*, 1981). Plasmid-carrying strains are thought to display decreased growth rates because of the energy expended and the carbon sources needed for both maintenance and replication of the plasmid, and its functions (Goodwin & Slater, 1979).

Previous studies of the competitive fitness cost engendered by plasmid carriage have focused on common laboratory plasmids or cloning vectors (Dahlberg & Chao, 2003; Bjorkman & Andersson, 2000). There appears to be no studies to date that have determined fitness costs associated with plasmids from veterinary isolates.

The aim of this chapter is to determine whether the carriage of plasmids pUK2001, pUK2002 and pUK2003 confer detectable competitive fitness costs on a host bacterium, either in single culture experiments (5hr growth rate comparisons) or during direct competition (for 5hr and for 5 days).

7.2 Materials and Methods

7.2a Bacterial strains and plasmids

The competitive fitness costs of the apr^R plasmids pUK2001, pUK2002 and pUK2003, identified in Chapter 5 were studied. Plasmid pUK2002 was selected for a more detailed analysis of growth rates of plasmid-carrying and plasmid-free strains. This plasmid was chosen because its size and transfer frequency was intermediate among the three plasmids isolated. Plasmids were maintained in *E. coli* MG1655 and *E. coli* J53. The *E. coli* K12 strains J62-2 (*lac*$^-$, *pro*$^-$ *trp*$^-$ *his*$^-$) and MG1655kan (Δ *lac*, Km) were used as isogenic competitors in J53 competitions and in MG1655 competitions, respectively.

5.2b Five hour growth rate comparisons

Cultures of each strain (plasmid-harbouring J53 or MG1655 and plasmid-free J53 or MG1655) were grown in 5ml Lennox L broth (LB) (Invitrogen) or Davis Mingioli broth supplemented with 0.4% glucose (designated MM for minimal media), for 16-18h at 37°C with shaking at 150 rpm. Starter cultures were diluted 1:10 in saline and used to inoculate 10mL LB or MM to give an initial density of approximately 1×10^6 cfu/mL. During incubation at 37°C with shaking at 150 rpm, samples were taken at 30 min intervals since initial inoculation for five hours. Cell densities were estimated by serial dilution in saline and selective plating. To determine whether plasmid segregation was occurring during the course of the experiment, viable counts of plasmid-harbouring strains were plated onto MacConkey plates, with or without 16mg/L apramycin.

7.2c Five hour competition study

The same protocol as that described for the five hour growth rate comparison was used to study plasmid-carrying and plasmid-free strains in direct competition, except that MG1655kan and J62-2 were used as the plasmid-free competitors. This was to enable the identification of plasmid-carrying, plasmid-free and transconjugant strains during the experiment. Starter monocultures were diluted 1:10 in saline and used to inoculate 10ml LB or MM at a ratio of 1:1 and an initial density of 1 x 10^6 cfu/mL of each strain. The formation of any transconjugants was measured by serial dilution and plating onto selective MacConkey plates. Supplementation with 40μg/mL apramycin and 32μg/mL kanamycin was used for MG1655 competitions, and 32μg/mL apramycin and 32μg/mL rifampicin for J53 and J62-2 competitions.

7.2d Five day competition experiments

Five-day competition experiments between plasmid-carrying MG1655 and plasmid-free MG1655kan were performed in MM to limit the frequency of plasmid transfer. Overnight cultures of the competing strains were grown up in separate bijoux containing 5mL MM. The two cultures were mixed in a 1:1 ratio (mix0) and used to inoculate 250μL of broth in each of six wells of a microtitre plate, to give an initial inoculum of approximately 1 x 10^6 cfu/mL. Initial counts of plasmid-carrying MG1655 and plasmid-free MG1655kan were determined by serial dilution and plating onto S-gal plates (Sigma). The presence of any transconjugants was detected by plating 100μL mix0 onto MacConkey plates supplemented with 40μg/mL apramycin and 32μg/mL kanamycin. Microtitre plates were incubated at 37°C with gentle shaking (150 rpm.). After 24 hours of growth, cultures were diluted 1:100 into fresh media. This dilution step was repeated daily for a total of five transfers. The densities of plasmid-free, plasmid-harbouring and transconjugant cells were estimated every 24 hours by serial dilution and selective plating. S-gal was used for the estimates of plasmid-carrying (black) and plasmid-free cells (white), and

MacConkey plates supplemented with 40mg/L apramycin and 32mg/L kanamycin for the quantification of transconjugants.

Relative fitness (S) was estimated as described by Lenski *et al* (1991)

$$S_{ij} = \left\{ \frac{Ln\left[N_i(1) / N_i(0) / Ln(2)\right]}{Ln\left[N_j(1) / N_j(0) / Ln(2)\right]} \right\} - 1$$

Where $N_i(0)$ and $N_j(0)$ are the initial densities of the tested clone and the common competitor (MG1655kan) respectively, and $N_i(1)$ and $N_j(1)$ are their corresponding final densities.

Fitness was estimated after 24 hours of direct competition and also after five days of direct competition (with a 1:100 dilution of each culture into fresh broth after each 24 hour period). Some workers have calculated fitness only after 24 hours (Dahlberg & Chao, 2003). The reason for estimating fitness after 24 hours and after five days of competition was to determine if the estimates differed with longer competition times. In all assays, the relative fitness was calculated as the growth of MG1655 relative to MG1655kan. In this way a positive relative fitness calculated as described by Lenski *et al.* (1991) demonstrates a growth advantage of MG1655 compared to MG1655kan.

The selection rate constant, r (day^{-1}) was calculated by regressing the natural log-transformed ratio of plasmid-bearing to plasmid-free cell densities against time (Lenski *et al.*, 1994), over the five day time period.

7.2e Reproducibility of viable count method

It was hypothesised that the differences between growth rates of plasmid-carrying and plasmid-free strains may be slight, if detectable. It was important therefore, to determine the level of variation introduced by the viable count method used to quantify the numbers of bacteria present. Four colonies of *E. coli* 10418 were

inoculated into 5mL LB and grown for 8 hours at 37°C with shaking at 150 rpm. The culture was then diluted 100,000 fold in saline. To study the variation with repeat dilutions, thirty 100μL aliquots were diluted 10-fold in saline and plated onto MacConkey plates for quantification (method A). To study variation within one dilution (method B), a 1mL aliquot was diluted 10-fold in 9mL saline, and 30 aliquots of this dilution were subsequently plated out onto MacConkeys plates.

7.2f Statistical analyses

The effects of plasmid, host, media (LB or MM) and detection plate (MacConkeys or MacConkeys supplemented with 16mg/L apramycin) on the number of cfu/mL during the five-hour growth experiments and five-day competition assays were analysed with generalised linear mixed effects models. Data that were not normally distributed were log-transformed before the analysis was performed. Different flasks or wells were entered as a random effect to account for both the variation between replicate experiments and the lack of independence between samples. Type of detection plate was included in the model as a fixed effect to determine whether plasmid segregation was occurring in any of the experiments.

A second linear mixed effects model was run to study the effect of plasmid carriage during the five-hour growth experiments. Non-significant interactions were removed from the previous models, and time, host (J53 or MG1655), media (LB or MM) and plasmid carriage were entered as fixed effects. Different flasks were again entered as a random effect.

7.3 Results

7.3a Variation introduced by the viable count method

Thirty replicate dilutions of *E. coli* 10418 (method A) and 30 replicate aliquots of the same diluted *E. coli* 10418 culture (method B) were plated onto MacConkeys plates for quantification by the viable count method. Figure 7.1 shows the spread of counts obtained with each method. The mean count for each method is highlighted on the graph.

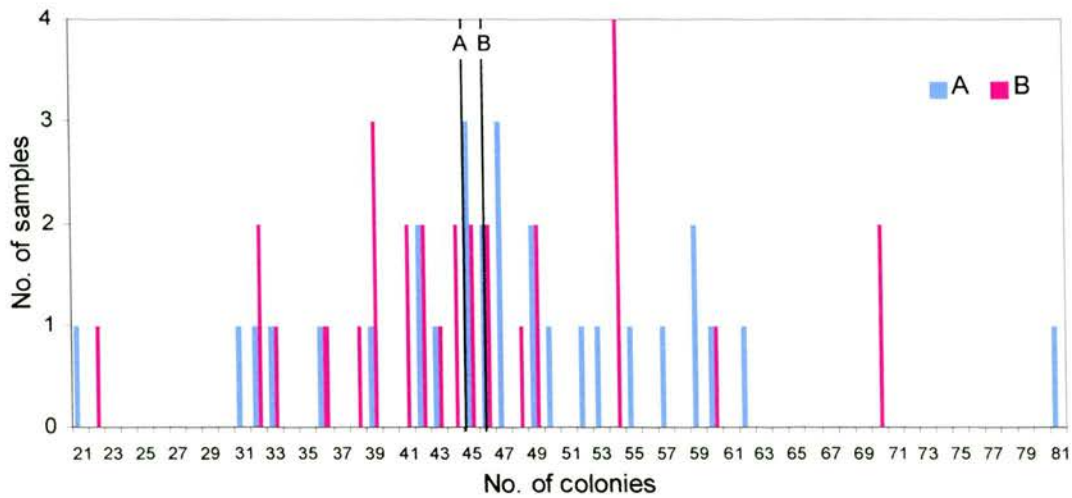


Figure 7.1 Spread of counts obtained when repeat dilutions of the same bacterial culture was diluted and plated out 30 times (blue bars; method A), and when 30 repeat aliquots of the same culture were plated out (pink bars; method B).

Figure 7.1 shows that although all replicate counts were performed on dilutions of the same 8-hour bacterial culture, a variety of counts are obtained. The counts were not significantly different between the two methods ($t_{58} = 0.54$, $P = 0.591$), but ranged from 21 to 81. If the colony counts of two samples differed by less than 60 colonies, it would be difficult to state whether the two samples contained significantly different cfu/mL. This is because the variation observed may be a result

of variation introduced by the methodology. Table 7.1 lists the descriptive statistics of the data sets from each method.

Statistic	Method	
	A	B
No. of replicates	30	30
Mean	46.6	45
Standard Error	2.2	1.9
Standard Deviation	12	10.4
Coefficient of variation	0.26	0.23
Range	60 (21-81)	48(22-70)

Table 7.1 Descriptive statistics of data from method A and method B.

The range of counts, standard error and standard deviation indicate that method A (multiple dilutions of the same culture) generates slightly more diversity in the colony counts than multiple aliquots of a diluted culture (method B). The variation between the two methods was insignificant ($F = 1.55$, $P = 0.248$). From the results described in Figure and Table 7.1, it was decided that a difference of at least 60 colonies should be present between identical dilutions of two given cultures, to be certain that they had different cfu/mL of bacteria.

7.3b Five-hour growth rate comparisons

Firstly, the growth curves of each strain (MG1655pUK2002, MG1655kan, J53pUK2002 and J53) in LB and MM was plotted (at least five replicates) to determine the phases of growth exhibited under these conditions. The growth curves of replicate cultures in LB are shown in Figure 7.2, and the geometric means of each of these, in Figure 7.3. Similarly, the growth curves of replicate cultures in MM and the geometric means of these results are shown in Figures 7.4 and 7.5 respectively.

The missing data points on the graph occur when the number of cfu on a plate was of an uncountable number (>300cfu). The exclusion of these data points does not affect

the ability of the linear mixed effects model to determine the significance of any observed trends.

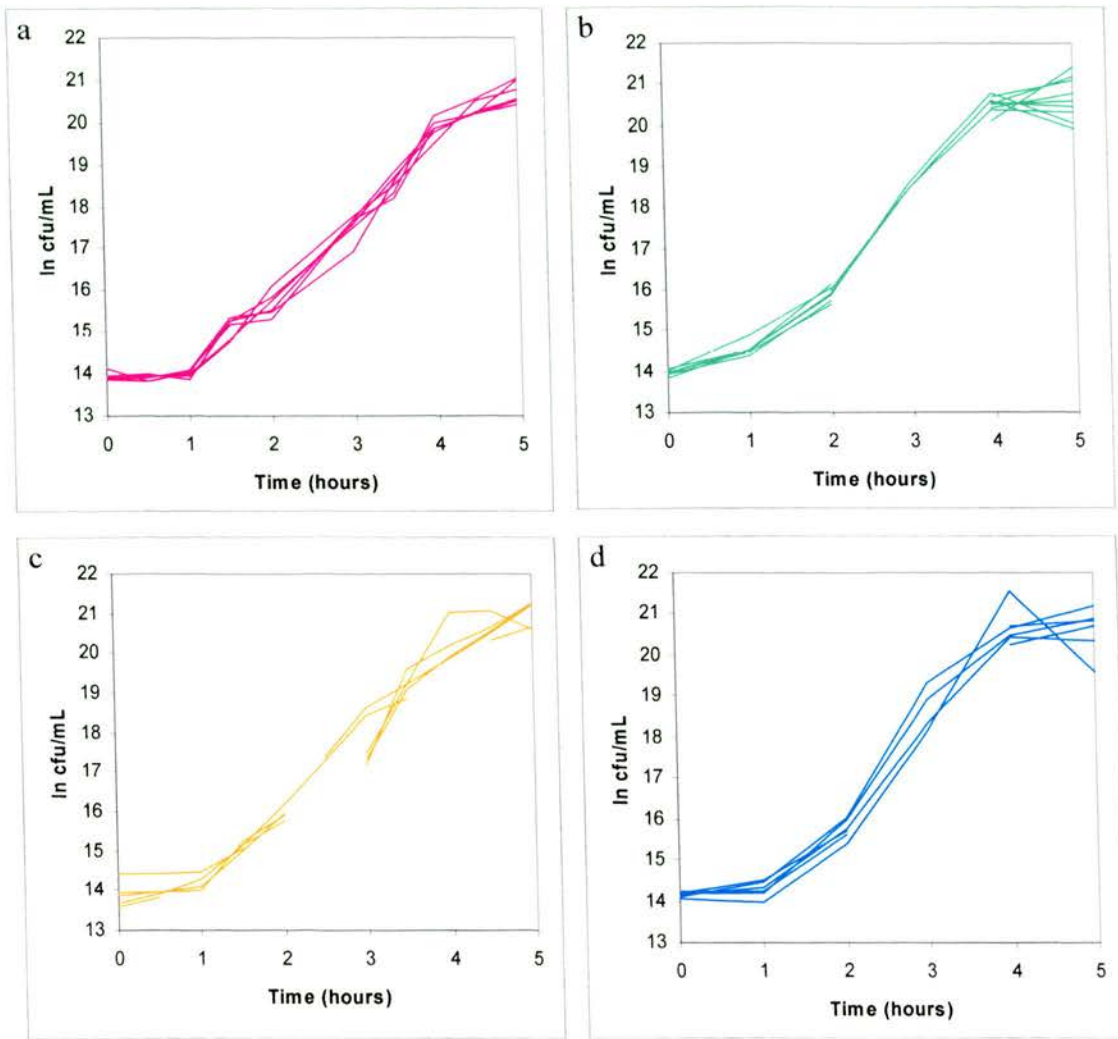


Figure 7.2 Replicate growth curves of a, MG1655pUK2002; b, J53pUK2002; c, MG1655kan; and d, J53 in 10mL LB.

In Figure 7.2 it can be seen that the replicate experiments show similar trends overall, but there are times during the experiment where more variation is present. During the first hour, more variation between the five replicates of MG1655kan is observed than at 1.5 and 2 hours. This may result in inaccurate estimates of growth rates if they are measured from the moment of initial inoculation (t = 0), as the slopes

of each replicate of MG1655kan during the first hour are quite different. In the graphs of J53pUK2002 and J53 (b and d respectively) replicate cultures, the slopes of each replicate appear very similar except at 4 and 5 hours. Here, the viable counts of some replicates continue to increase where in other replicates they either decrease or reach a stationary phase with no overall increases in viable cell numbers. Overall, it appears that variation between replicate growth curves is most evident during the first hour and after the fourth hour of growth.

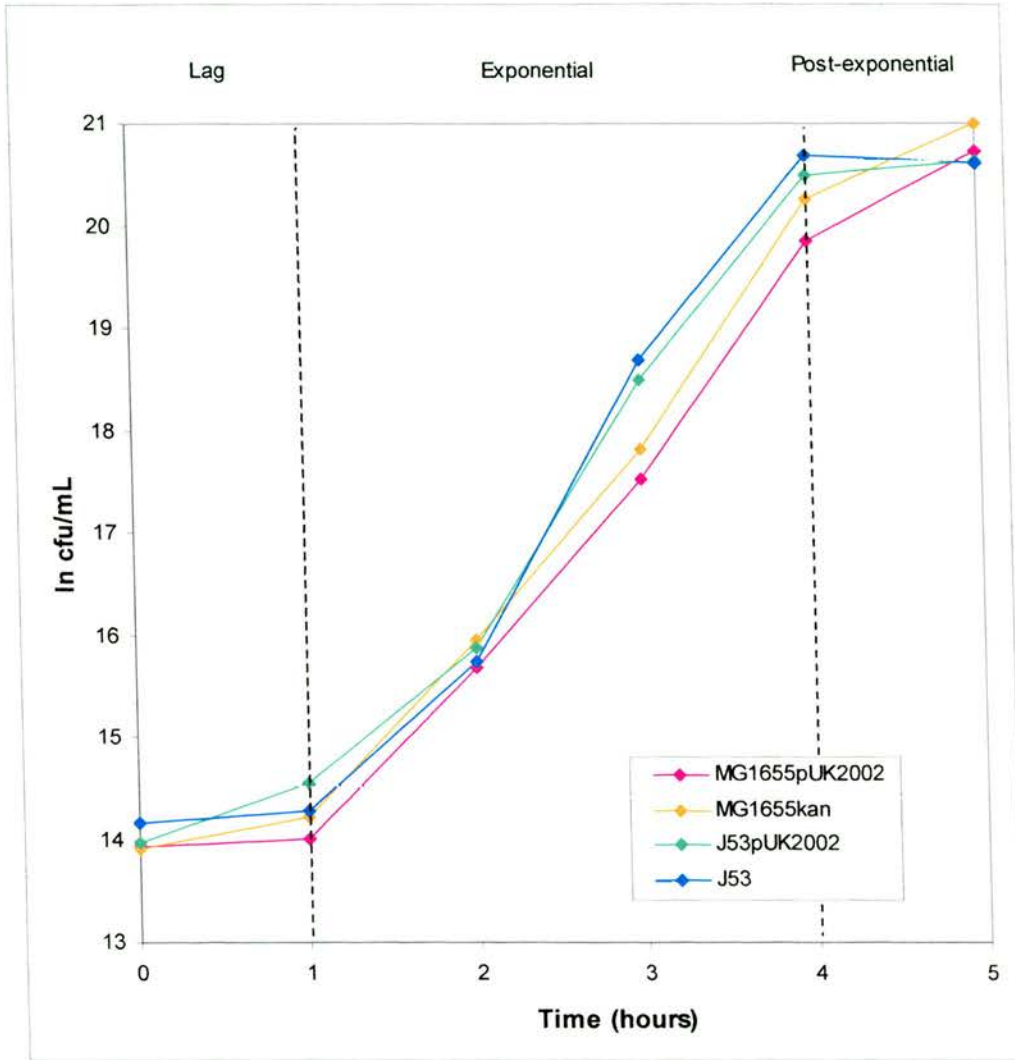


Figure 7.3 Geometric mean growth curves of MG1655pUK2002, MG1655kan, J53pUK2002 and J53 in 10mL LB.

In the comparison of the geometric mean growth curves of MG1655pUK2002, MG1655kan, J53pUK2002 and J53 (Figure 7.3), all four cultures demonstrate a one

hour period of reduced growth in comparison to the exponential phase occurring between one and four hours. The decreased growth rate is more evident with cultures of MG1655pUK2002 and J53. After four hours, the cultures enter a post-exponential growth phase, where J53pUK2002 and J53 cultures show a more dramatic decrease in growth rate than MG1655pUK2002 and MG1655kan. In this way, after four hours the growth curve varied in a manner dependent on the host strain (J53 or MG1655).

With respect to determining differences in growth rates between plasmid carrying and plasmid free strains, it is important to define periods of growth that are most comparable. From Figure 7.2, it is clear that less variation between replicate growth curves is present during one and four hours. Between the four different strains, obvious variation was present during the lag and post-exponential growth phases. In the post-exponential phase, the pattern of growth appeared dependent on host strain. Therefore, only data from the exponential phase (between one and four hours) was used to compare growth rates between plasmid-free and plasmid-carrying strains.

The increase in cfu/mL over time was host specific, with MG1655 cultures (irrespective of plasmid carriage) containing significantly less cfu/mL with time (slower growth) than J53 strains, $t_{50} = -4.17$, $P = 0.0001$. Plasmid segregation was discounted as changes in cell numbers over time revealed no significant difference in the recovery of plasmid-carrying cells on MacConkey plates or on MacConkey plates supplemented with 16mg/L apramycin, $t_{50} = 0.59$, $P = 0.56$

In MM more variation between replicate experiments was apparent (Figure 7.4) than between replicates in LB (Figure 7.2). This is likely to be because of variation introduced when making the MM, as each flask was made up separately from double concentrate DM, distilled water and glucose. Slower growth was predicted in MM than in LB, and this is what is observed, with the presence of <18 Ln cfu/mL after five hours compared to around 21 Ln cfu/mL in LB. The J53pUK2002 starter culture may have contained more cells than the other starter cultures, because the cultures at time 0hr contain more cfu/mL than the other three growth curve experiments.

The geometric mean growth curves in MM (Figure 7.5) show less well-defined growth phases compared to those in LB (Figure 7.3). The growth curves of MG1655kan and J53 have much more distinct lag phases than those of the plasmid-carrying strains (MG1655pUK2002 and J53pUK2002). None of these curves reach a post-exponential phase, which was observed in LB.

To keep the two data sets (LB growth and MM growth) as comparable as possible, again only data between one and four hours were used in the statistical analysis

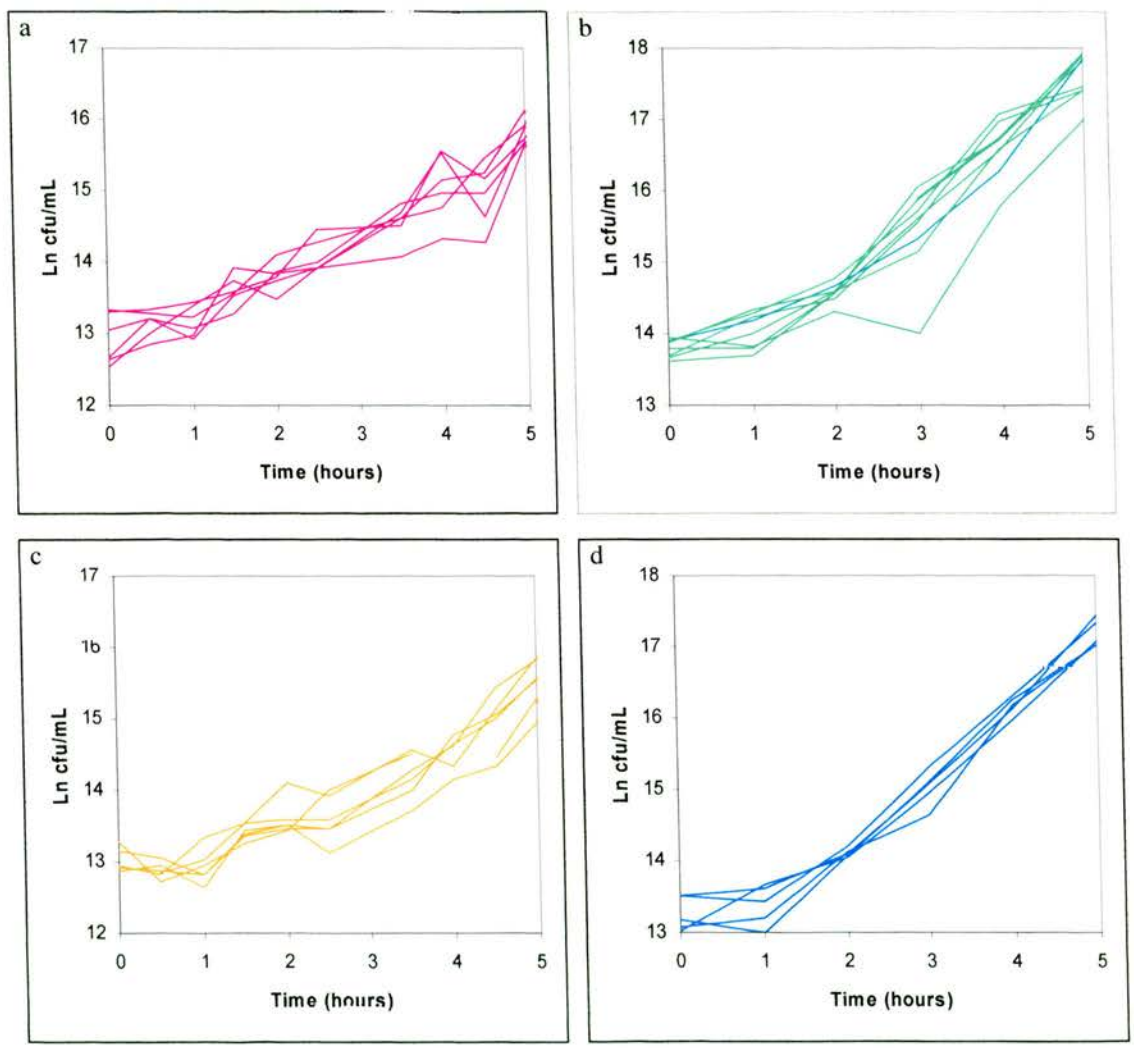


Figure 7.4 Replicate growth curves of a, MG1655pUK2002; b, J53pUK2002; c, MG1655kan; and d, J53 in 10mL MM.

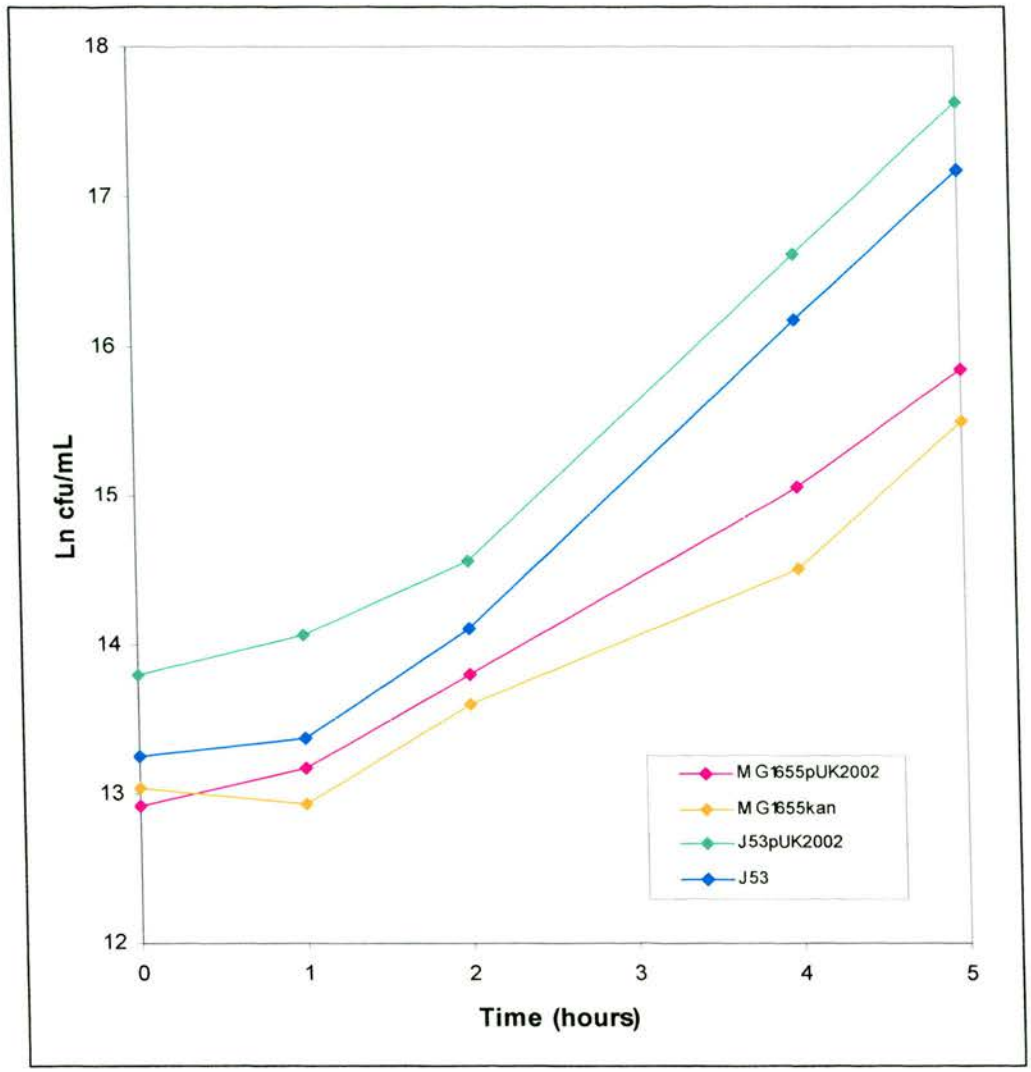


Figure 7.5 Geometric mean growth curves of MG1655pUK2002, MG1655kan, J53pUK2002 and J53 in 10mL MM.

In MM, cultures of J53 (with or without plasmid) contained significantly more cfu/mL overall, during the course of the experiment than cultures of MG1655 (with or without plasmid) $t_9 = -2.41$, $P = 0.039$. The increase in cfu/mL with time was host specific, with J53 cultures growing significantly faster than MG1655 cultures, $t_{61} = -2.16$, $P = 0.035$. Plasmid segregation was again discounted as changes in cell numbers over time revealed no significant difference in the recovery of plasmid-carrying cells on MacConkey plates or on MacConkey plates supplemented with 16mg/L apramycin, $t_{57} = -92$, $P = 0.36$.

In the final model the growth of J53, J53pUK2002, MG1655kan and MG1655pUK2002 were compared. The number of cfu/mL in each culture of plasmid-carrying strains (J53pUK2002 and MG1655pUK2002) throughout the course of the experiment did not differ significantly from those of plasmid-free cultures, $t_{31} = 1.35$, $P = 0.19$. Plasmid carriage did not affect the changes in cfu/mL over time (i.e. cell growth), $t_{171} = 0.363$, $P = 0.7172$. The increase in cfu/mL over time was host-dependant, with MG1655 cultures growing significantly slower than J53 cultures, t_{176}, $P = < 0.0001$.

7.3c Five-hour growth in direct competition

When plasmid-carrying (J53pUK2002 and MG1655pUK2002) and plasmid-free (J53 and MG1655kan) strains were grown in direct competition, the mean colony counts of competing strains did not differ by > 60 colonies. This suggests that plasmid carriage did not affect growth because this level of variation could occur on account of the methodology. When tested statistically, overall fewer cfu/mL were present in MG1655 cultures (either plasmid-carrying or plasmid-free MG1655) than J53 cultures, $t_{12} = -3.86$, $P = 0.002$, but the growth rates of MG1655 strains and J53 strains were not significantly different, $t_{245} = 0.94$, $P = 0.35$.

Plasmid carriage did not affect the numbers of cfu/mL over the course of the experiments, $t_{243} = -0.14$, $P = 0.89$, and did not affect the growth rates of the host strain (increase in cfu/mL with time), $t_{243} = 1.06$, $P = 0.29$. Plasmid segregation was discounted in both LB and MM, as changes in cfu/mL with time were not significantly different when measured on MacConkey plates and MacConkey plates supplemented with 16mg/L apramycin, $t_{46} = -0.2$, $P = 0.85$ (LB data), and $t_{70} = -1.19$, $P = 0.24$ (MM data).

7.3d Five-day growth in direct competition

Relative fitness estimates and selection rate constants of plasmid-carrying MG1655 in direct competition with MG1655kan are listed in Table 7.2. The control for this 5-day competition assay was the direct competition of MG1655 and MG1655kan. Relative fitness estimates of $S_{ij} = 0.005 \pm 0.005$ after 24 hours revealed that the insertion of the kanamycin resistance gene into MG1655 marginally reduced the competitive fitness of the strain (Table 7.2). The relative fitness cost of the kanamycin resistance gene is more evident after five days of competition.

If there is no difference in the growth rate of two competing cultures but one has a higher inoculum, the difference in competing cell densities may be amplified with each repeat passage. To ensure that variation the number of bacteria present in the initial cultures did not influence the outcome of the competition, control experiments were repeated with an initial ratio of 1:2 and of 2:1 MG1655 to MG1655kan. The selection rate constants of six replicate competitions did not differ significantly when initial ratios of 1:2 or 2:1 were used ($t_6 = -0.386$, $P = 0.712$).

Table 7.2 Relative fitness estimates and selection rate constants of MG1655 harbouring plasmid pUK2001, pUK2002 or pUK2003, in direct competition with MG1655kan.

Plasmid	Replicate	Selection rate constant (r) (day^{-1})[a]	Relative fitness Sij[b] 24h	5 day
pUK2001	1	0.253	0.145	0.290
	2	0.219	0.149	0.236
	3	0.293	0.295	0.405
	4	0.330	0.188	0.593
	5	0.107	0.144	0.263
	6	0.136	0.211	0.319
	mean	0.223 ± 0.088	0.189 ± 0.024	0.351 ± 0.054
pUK2002	1	0.358	0.140	0.638
	2	0.378	0.182	0.361
	3	0.190	0.350	0.332
	4	0.215	0.143	0.301
	5	0.351	0.199	0.405
	6	0.511	0.124	0.437
	mean	0.334 ± 0.117	0.190 ± 0.034	0.412 ± 0.049
pUK2003	1	-0.0839	0.003	-0.071
	2	-0.0915	0.015	-0.043
	3	-0.1261	0.029	-0.072
	4	-0.0361	0.016	-0.056
	5	0.0065	ND	-0.041
	6	-0.0601	ND	-0.057
	mean	-0.069 ± 0.044	0.016 ± 0.01	-0.057 ± 0.01
Control	1	0.0567	0.016	0.077
	2	0.0047	0.008	0.059
	3	0.0295	-0.009	0.014
	4	0.0704	0.004	0.052
	5	0.0682	ND	0.058
	6	0.0051	ND	0.000
	mean	0.0391 ± 0.0302	0.005 ± 0.005	0.043 ± 0.012

[a] Standard deviation is given for each mean estimate, [b] standard error is given for each mean estimate.

Table 7.2 shows that pUK2001 and pUK2002 confer fitness advantages on MG1655 that are far greater than the advantage of this strain over its kanamycin resistant counterpart. The fitness advantages conferred by carriage of these plasmids are more evident after five days of competition. The mean colony counts of the raw data showed a difference of > 60 colonies after day three and day one for pUK2001 and pUK2002 respectively. The selection rate constants for MG1655 carrying these plasmids were significantly different from the control (pUK2001; $t_{10} = -4.87$, $P =$

0.0006, pUK2002; $t_{10} = -5.96$, $P = 0.0001$). Plasmid segregation of pUK2001 or pUK2002 was discounted, as changes in cfu/mL with time did not differ significantly when measured on S-gal plates or MacConkey plates supplemented with 16mg/L apramycin ($t_{45} = 0.96$, $P = 0.342$, $t_{112} = 1.18$, $P = 0.24$ respectively). Over the five days of competition, cfu/mL of MG1655pUK2001 differed significantly from those of MG1655kan ($t_{51} = -1.24$, $P < 0.0001$). Cfu/mL of MG1655pUK2001 increased significantly with time ($t_{23} = 4.67$, $P = 0.0001$).

The increase over time of cfu/mL of MG1655pUK2002, compared to MG1655kan was significant, $t_{119} = -11.133$, $P = <0.0001$. When separate models of the MG1655pUK2002 or the MG1655kan data were run, the cfu/mL of MG1655kan decreased significantly over time, $t_{57} = -12.89$, $P <0.0001$.

In contrast, pUK2003 appeared to confer a fitness cost which was detectable after five days of competition (Table 7.2). After only 24 hours of competition, the difference in relative fitness was not significant ($t_9 = -1.533$, $P = 0.176$). Fewer cfu/mL of MG1655pUK2003 compared to cfu/mL of MG1655kan were detected during the course of the assay, but the difference was not significant ($t_{33} = -0.92$, $P = 0.36$). Cfu/mL of MG1655pUK2003 decreased with time, but this decrease was not significant ($t_{14} = -0.72$, $P = 0.48$). Consistent with these statistical findings, the colony counts of competing strains throughout the five-day competition did not differ by > 60 colonies.

7.4 Discussion

Resistance plasmids are threatening the successful management of both human and animal bacterial infections. Traditionally, the carriage of resistance plasmids is thought to engender a competitive fitness disadvantage on the host bacterium, in the absence of selective pressure by antibiotics (Goodwin & Slater, 1979; Lee & Edlin, 1985; Noack *et al.*, 1981; Zund & Lebeck, 1980). In this way, the presence of plasmid-mediated resistance may be limited by restricting the use of the selective

antibiotics, allowing sensitive strains to outcompete the resistant bacteria. A few reports have documented the amelioration of a fitness disadvantage conferred by the presence of chromosomal mutations (Bjorkman *et al.*, 1998; Reynolds, 2000), transposable elements (Hartl *et al.*, 1983; Modi *et al.*, 1992) conjugative and non-conjugative plasmids (Bouma & Lenski, 1988; Enne *et al.*, 2004b; Negri *et al.*, 2000). Very few of these reports have studied naturally occurring plasmids, focussing mainly on cloning vectors and common laboratory plasmids (Bjorkman & Andersson, 2000). There appears to be no studies of fitness costs associated with plasmids isolated from veterinary isolates. The apr^R plasmids identified in the commensal *E. coli* of the calves sampled in this study were large (> 90kb), and so the presence of a fitness disadvantage associated with the carriage of these plasmids was expected.

Previously, the biological cost of resistance has been measured experimentally by estimating growth rates of sensitive and resistant bacteria in monocultures (Bjorkman *et al.*, 1998), or by pairwise competition studies in batch cultures (Dahlberg & Chao, 2003; Enne *et al.*, 2004b; Lenski *et al.*, 1994), chemostats (Helling *et al.*, 1981, Modi *et al.*, 1992) or experimental animals (Bjorkman *et al.*, 1998; Enne *et al.*, 2004a; Negri *et al.*, 2000). In this chapter growth rate comparisons of monocultures and competing cultures were compared, in addition to pairwise competition studies in batch culture.

The growth of isogenic strains with and without pUK2002, in single culture experiments and during direct competition, did not reveal any significant differences in growth rates. Similarly, Enne *et al.* (2004b) did not find significantly shorter generation times of plasmid-carrying strains compared to their isogenic plasmid-free counterparts. The use of linear mixed effects models to analyse the data, enabled more information to be gleaned than by comparing growth rates by paired t-tests, in that interactions between factors such as media and host strain could be analysed. This meant it was possible to account for any differences for example, between the host strain used, when studying the effect of plasmid carriage. The growth of isogenic plasmid-carrying and plasmid-free strains for five hours, either in direct

competition or in single cultures may be too short a period for any slight differences in growth rate to be detected. A five-day pairwise competition was performed to test this hypothesis.

Different fitness cost estimates have been used to quantify the difference in competitive fitness between two strains (Lenski *et al.*, 1991). These include estimates of relative fitness (Helling *et al.*, 1981; Reynolds, 2000), per generation fitness impact (Enne *et al.*, 2004a, 2004b), generation times (Bjorkman *et al.*, 1998) and selection rates or coefficients (Dahlberg & Chao, 2003; Lenski *et al.*, 1994; Modi *et al.*, 1992; Negri *et al.*, 2000). The lack of a standard estimate to quantify fitness costs leads to difficulties in comparing results between studies. In this chapter, both the selection coefficient and the relative fitness (as described by Lenski *et al.*, 1991) were used to allow the results to be compared with those of similar published studies.

During five days of pairwise competition, the average selection coefficient of pUK2002 was found to be $r = 0.334$ day $^{-1} \pm 0.117$. Similar selection coefficients ($r = 0.216$ day$^{-1} \pm 0.043$) have been described by Lenski *et al.* (1994) for a 2.9kb plasmid conferring tetracycline resistance. The authors suggest that the tetracycline resistance gene (*tetC*) was the factor responsible for the enhanced fitness with plasmid carriage in the co-evolved host. Interestingly, plasmids pUK2002 and pUK2003 also carry a tetracycline resistance determinant (*tetB*). Plasmid pUK2003 did not confer a fitness advantage, and demonstrated a potential fitness cost ($r = -0.069 \pm 0.044$ day $^{-1}$) although more replicate experiments and longer competition times would be required to improve the significance of this result, so this gene may be unrelated to the fitness advantage of pUK2002.

The competitive fitness advantage of pUK2001 and pUK2002 was observed in hosts that had no evolutionary history of association. This is a surprising result because the competitive fitness advantage described by Lenski *et al.* (1994) was only present when the plasmids and hosts had coevolved for 500 generations. In contrast, Enne *et al.* (2004b) report a fitness advantage in both naïve and adapted hosts, of a 6.2kb plasmid conferring sulphamethoxazole and streptomycin.

The relative fitness of pUK2001 and pUK2002 ($S = 0.189 \pm 0.024$ and 0.190 ± 0.034 respectively) after 24 hours of competition are within the range of mean fitness estimates of R1- or RP4-bearing J53 after 24h following approximately 1100 generations of growth in an antibiotic-free environment (range of 0.16 to 0.35) (Dahlberg & Chao, 2003).

The five-day pairwise competition study described in this chapter has an advantage over the five-hour competition study in that more components of bacterial growth are compared. For example, differences in initial lag phase, speed of growth in exponential phase, and efficiency of resource utilisation. Over 24 hours, all available resources are used up and the cells enter stationary phase. Upon subculture into fresh broth, this complete cycle of growth is repeated. A substantial amount of variation occurred between the replicate competition experiments described in this chapter, demonstrating the necessity for repeat estimates of fitness.

The results of this chapter indicate that pUK2001 and pUK2002 confer a competitive fitness advantage on the naïve host strain MG1655, under the conditions tested (minimal media, incubation at 37°C with shaking at 150 rpm.). At present the nature of this advantage is unknown. The plasmids may carry a metabolic function aiding growth of the host strain under the conditions tested. To determine whether a gene present on the plasmid is responsible for conferring a competitive advantage will require sequencing of the plasmids.

It is also unclear at this stage whether the competitive advantage observed *in vitro* will also occur *in vivo*. Although an attempt was made to stress the cells by growing them under nutrient limited conditions, this is unlikely to be as stressful as conditions *in vivo*. Intestinal *E. coli* must compete for nutrients against many other intestinal micro-organisms (bacteria; *Bacteroides* spp. *Klebsiella, Proteus, Streptococcus faecalis*; the yeasts *Candida* and *Torulopsis*; and protozoa; *Entamoeba*) (Stanier *et al.*, 1983), some of which may produce bacteriocins (Avelar *et al.*, 1999). Gut bacteria are likely to be exposed to cycles of nutrient starvation and abundance *in*

vivo (Hardy, 1984). The carriage and maintenance of a large accessory genetic element is likely to require more of the cells available energy, than to not carry it. During periods of starvation, when the cell must reduce its metabolic activity in order to survive, strains without the plasmid are likely to overgrow those with a plasmid unless there is direct selection for its presence. Other conditions may arise when it is more advantageous to carry such a plasmid. However, on consideration of the occurrence of these apr^R plasmids (which required selective isolation for their detection), they are rare in the commensal population studied, and so it is likely that a selective pressure not yet identified is responsible for their presence. This may be co-selection by tetracycline or streptomycin farm usage for pUK2002, or the use of disinfectants or feed supplements, as is discussed in Chapter 5.

Although the fitness advantage described in this chapter should be interpreted with caution until *in vivo* studies have been performed, this work demonstrates an *in vitro* competitive fitness advantage associated with the carriage of pUK2001 and pUK2002 that is comparable to other published studies.

Chapter 8. The search for extended-spectrum beta-lactamases

8.1 Introduction

In 1965 the first plasmid-mediated β-lactamase in a gram-negative organism, was described (Datta & Kontomichalou, 1965). This enzyme was designated TEM-1 after the Greek patient, Temoniera, from whom it was isolated. TEM-1 efficiently hydrolyses amoxycillin, and is commonly found on transposons (Yamamoto *et al.*, 1982) and plasmids (Gulay *et al.*, 2000; Simpson *et al.*, 1980). Because of the mobility of these genetic elements, the enzyme TEM-1 has been able to spread to other species of bacteria, such as *Klebsiella* (Jeong *et al.*, 2004a), *Proteus* (Chanal *et al.*, 2000), *Salmonella* (Rivera *et al.*, 1991), *Pseudomonas* (Calderwood *et al.*, 1982) and *Acinetobacter* (Vila *et al.*, 1993). TEM-1 has since spread worldwide, and is currently the most common plasmid-mediated β-lactamase (Amyes *et al.*, 1996). A sulphydryl variable of this enzyme (SHV-1), originally isolated from *Klebsiella pneumoniae* is also commonly found amongst Enterobacteriacae (Heritage *et al.*, 1999). This enzyme is usually plasmid mediated in *E. coli* isolates (Bradford, 2001).

Extended-spectrum β-lactams were developed to combat organisms harbouring β-lactamases. These are based on the original β-lactam structure but with additional side-chains intended to prevent their hydrolysis. However, each new extended-spectrum β-lactam used therapeutically has been met with the discovery of a new β-lactamase (designated extended-spectrum β-lactamases or ESBLs) capable of its hydrolysis. Most ESBLs are derived from TEM or SHV enzymes, by the presence of between one and five point mutations enabling the extended-spectrum β-lactams antibiotics to fit into the active site of the enzyme for subsequent hydrolytic inactivation (Bush *et al.*, 1995). Other classes of ESBL include those that are based on CTX-M and OXA enzymes. The latter is commonly found in Pseudomonas isolates (Danel *et al.*, 1999; Hall *et al.*, 1993; Mugnier *et al.*, 1998), but CTX-M enzymes have been isolated mainly from salmonella (Edelstein *et al.*, 2004; Tzouvelekis *et al.*, 2000) and *E. coli* strains (Briñas *et al.*, 2003b; Jeong *et al.*,

2004b). CTX-M enzymes preferentially hydrolyse cefotaxime and are becoming a huge problem (Pitout *et al.*, 2004; Pournaras *et al.*, 2004). ESBLs are commonly encoded by self-transmissible or mobilisable plasmids, which further exacerbate the therapeutic challenges posed by these enzymes.

The presence of ESBLs in organisms causing infections is now a global problem. The prevalence of ESBLs amongst clinical isolates varies between countries and even within a country (Winkur *et al.*, 2001). In France 3.2% of 2506 Enterobacteriacae isolated in 1998 produced an ESBL (De Champs *et al.*, 2000). In contrast, a recent survey in Japan estimated that less than 0.1% of *E. coli* and 0.3% of *K. pneumoniae* strains isolated possessed an ESBL (Yagi *et al.*, 2000). The type of ESBL most commonly identified is also often country or region specific. In the United States TEM-10 producing organisms have caused several unrelated outbreaks (Bradford *et al.*, 1994; Schiappa *et al.*, 1996; Wiener *et al.*, 1999), but in Europe this enzyme is much less common (De Champs *et al.*, 2000; Perilli *et al.*, 2002). In Chicago, a common plasmid harbouring TEM-10 was found in 17 isolates from 20 hospitals or nursing homes (Wiener *et al.*, 1999). PFGE demonstrated that seven different *Klebsiella pneumoniae* and *E. coli* strains were carrying the plasmid, leading the authors to presume that the plasmid was present in the commensal flora of the patients. This interesting and alarming speculation is substantiated by other studies on β-lactam resistance in commensal organisms (Thomson *et al.*, 1993). In Turkey, a high incidence of ampicillin resistance has been found in commensal faecal *E. coli* from healthy volunteers (Gulay *et al.*, 2000). In South Africa, 94.3% of ampicillin resistant commensal faecal gram-negatives from healthy volunteers were found to harbour TEM-1 (Shanahan *et al.*, 1995).

Although extended-spectrum β-lactams are used in animal husbandry, there have been very few studies on the characterisation of β-lactamases from healthy food-producing animals (Briñas *et al.*, 2002; 2003b; Zhao *et al.*, 2001). ESBLs have been identified in pathogenic organisms from veterinary isolates (Briñas *et al.*, 2003a) but not yet in commensal isolates. This may be because therapeutic failures have not occurred or not been detected, and so the presence of ESBLs has not been sought.

Alternatively, the use of cephalosporins in animal husbandry may have not yet lead to the selection and development of resistant isolates. The aim of this chapter was to determine whether any of the commensal *E. coli* collected from the calf cohort, demonstrating reduced susceptibility to 3rd generation cephalosporins, harboured an ESBL. In addition, a *Salmonella enterica* serotype Enteritidis isolate (*S.* Enteritidis), identified by the Scottish Salmonella Reference Laboratory (SSRL) in Glasgow was included in the study because it was implicated in a Salmonellosis outbreak during which therapeutic failures with 3rd generation cephalosporins were reported. Because antibiotic resistance in relatively uncommon in *S.* Enteritidis (Vahaboglu *et al.*, 2001), the mechanism of resistance was of interest.

The two aims of this chapter are described as follows:

1. To determine if the cephalosporin resistant commensal *E. coli* isolated from the calves in this study harbour ESBLs.
2. To establish the mechanism of resistance of the *S.* Enteritidis isolate to 3rd generation cephalosporins.

8.2 Materials and Methods

8.2a Bacterial isolates

This study was carried out on 52 isolates of the amp^R population identified by work described in Chapter 3 as having reduced susceptibility to 3rd generation cephalosporins (cefotaxime MIC ≥ 2mg/L, ceftazidime MIC ≥ 4mg/L).

The *S.* Enteritidis isolate was collected from an outbreak of salmonellosis in a general hospital in Glasgow, Scotland. The epidemiology of this outbreak and the work completed by the Scottish Salmonella Reference Laboratory (SSRL) is outlined below.

8.2b Salmonella outbreak

The Salmonella outbreak occurred from the 20th December 2001 to the 21st January 2002, during which nine isolates were obtained from five patients with salmonella gastroenteritis, and two asymptomatic members of staff. The SSRL identified the isolates as *Salmonella enterica* serovar Enteritidis PT21, by agglutination to polyvalent antisera and phage typing. Isolates of *S.* Enteritidis from two patients and one member of staff were sensitive to fourteen common antibiotics. The isolates from the remaining patients and one member of staff were resistant to ampicillin and cefotaxime. Plasmid profiling and PFGE were used by the SSRL to demonstrate that these isolates were clonal and carried a plasmid of 95 kb. One isolate (020003) was used to determine the mechanism of resistance of the isolates.

8.2c Antibiotic sensitivity

Initial antibiotic susceptibility testing of 183 amp^R isolates was performed with a panel of 25 commonly used antibiotics (Chapter 3). The susceptibility of *S.* Enteritidis 020003 to ampicillin and cefotaxime was determined by the SSRL by disc diffusion.

MICs of the amp^R *E. coli* to amoxycillin, cefotaxime, ceftazidime, and these antibiotics in combination with clavulanic acid (in a ratio of 2:1 antibiotic to β-lactamase inhibitor), were determined following BSAC guidelines (Andrews, 2001). NCTC strains *E. coli* 10418, *S. aureus* 6571 and *E. coli* 11560 were used as controls. Susceptibility testing of *S.* Enteritidis 020003 to ampicillin, cefaclor, cefotaxime and ceftazidime was performed using Etest strips (AB Biodisc, Sweden) following the manufacturer's instructions.

8.2d Determination of the presence of an ESBL

The presence or absence of an ESBL was determined by Etest strips specific for the detection of ESBLs, containing cefotaxime or ceftazidime, with and without clavulanic acid, and by double disc synergy tests (Livermore & Brown, 2001). Discs containing 10μg cefpodoxime, 30μg ceftazidime or 30μg cefotaxime with and without clavulanic acid (1μg with cefpodoxime and 10μg with ceftazidime or cefotaxime) (Oxoid, Basingstoke, UK) were placed on IST agar plates (Oxoid) inoculated with 0.5 McFarland standard of test organism. Plates were incubated for 12-18 hours at 37°C. A positive result for the presence of an ESBL was recorded if the inhibition zone around the disc containing cephalosporin and clavulanic acid was ≥5mm larger than that around the disc containing only the cephalosporin.

NCTC organisms *E. coli* 11560 (TEM-1 +ve) and *E. coli* 10418 (ESBL –ve) were used as positive and negative controls respectively, for both the double disc synergy tests and Etest strips.

8.2e Amplification of $bla_{\text{TEM-1}}$ and $bla_{\text{SHV-1}}$

The ß-lactamase genes $bla_{\text{TEM-1}}$ and $bla_{\text{SHV-1}}$ were sought by PCR using a *Taq* polymerase kit (Promega) and the following oligonucleotide primer pairs: $bla_{\text{TEM-1}}$, 5'ATGAGTATTCAACATTTCCG-3', 5'-CCAATGCTTAATCAGTGAGG-3'; and $bla_{\text{SHV-1}}$, 5'-GCCCGGGTTATTCTTATTTGTCGC-3', 5'-CTTTCCGATGCCGCCGCCAGTCA-3'. DNA was initially denatured for 5 min at 95°C, followed by 30 cycles of amplification, and a final 10 min extension at 72°C. The amplification of $bla_{\text{TEM-1}}$ comprised a 30 sec denaturation at 95°C, 1 min of annealing at 53.3°C, and 1 min extension at 70°C. For $bla_{\text{SHV-1}}$ cycles of 30 sec denaturation at 95°C, 30 sec of annealing at 68°C, and 50 sec extension at 72°C, were used. Sequencing was performed by the chain termination method (Sanger *et al.*, 1977) in forward and reverse directions, and the sequences were compared with sequences in public databases.

8.3 Results

8.3a Antibiotic susceptibility

Initial breakpoint susceptibility testing revealed that 52 of 183 amp^R commensal *E. coli* tested, demonstrated reduced susceptibility to the 3rd generation cephalosporins cefotaxime (≥ 2mg/L) or ceftazidime (≥ 4mg/L). The MIC values of these isolates to amoxycillin, cefotaxime, ceftazidime, and these antibiotics in combination with clavulanic acid are shown in Figure 8.1. To display the MICs of the 52 isolates in a graph, isolates were sorted in ascending order according to firstly their amoxycillin MIC, then cefotaxime MIC, and final by their ceftazidime MIC. This was to aid the identification of isolates for further study. The isolates selected (n = 16) are highlighted on Figure 8.1 by black squares. These isolates were chosen to give a range of isolates representative of a group with similar sensitivity profiles, or because they were outliers in the general trend of the graph.

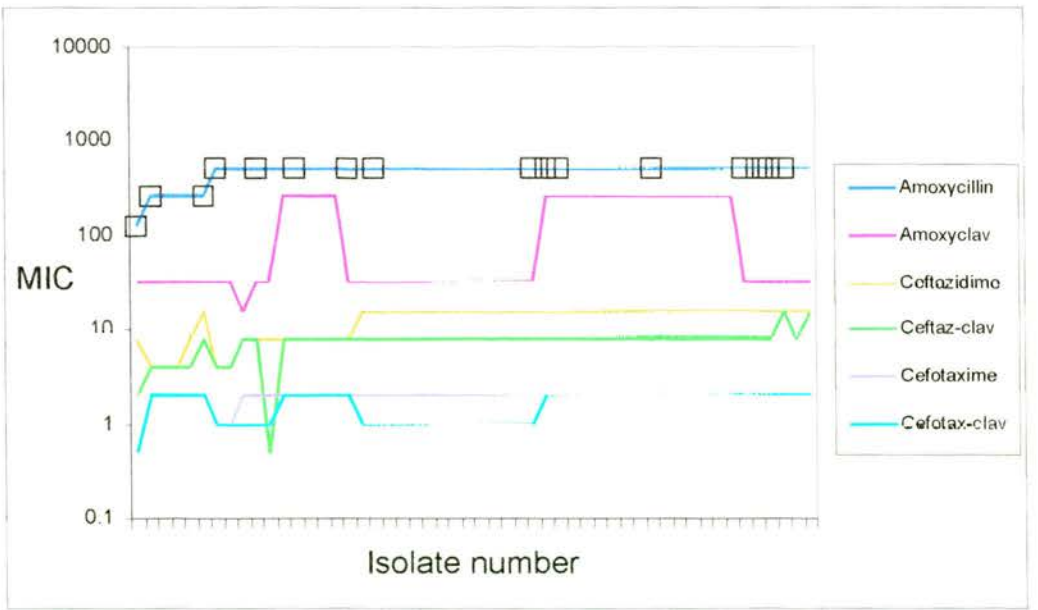


Figure 8.1 MICs of 52 isolates to amoxycillin, cefotaxime, ceftazidime, and these antibiotics in combination with clavulanic acid. Sixteen isolates were selected for further study. These are highlighted with black squares.

The susceptibilities of these 16 isolates to a range of cephalosporins and β-lactams are listed in Table 8.1. The breakpoint susceptibilities were determined as described in Chapter 3. The actual MIC values of amoxycillin, ceftazidime, cefotaxime, and these antibiotics in combination with clavulanic acid, are given in brackets next to the phenotype.

Table 8.1 Susceptibilities of 16 amp^{R} isolates selected for further study to β-lactams and cephalopsorins.

	Antibiotic (low and high breakpoints listed below)														
	AMP	AZL	PEN	AZT	THN	DNE	URX	FOX	TIZ	AMX	AMX/C	TAX	TAX/C	TAZ	TAZ/C
	4	6	0.12	2	4	8	8	8	2	4	2	2	2	4	4
	32	64	32	8	16	32	32	32	16	32	8	16	16	16	16
Isolate															
WF006962-4	R	R	R	I	R	R	I	I	S	R (128)	R (32)	S (0.5)	S (0.5)	I (8)	S (2)
WF006986-4	R	R	R	S	R	R	R	R	I	R (256)	R (32)	I (2)	I (2)	R (16)	I (8)
WF007204-4	R	R	R	I	R	R	I	I	S	R (256)	R (32)	I (2)	I (2)	I (4)	I (4)
WF007013-1	R	R	R	I	R	R	I	I	S	R (>256)	R (32)	S (1)	S (1)	I (4)	I (4)
WF006996-3	R	R	I	R	R	R	I	R	I	R (>256)	R (32)	I (2)	S (1)	R (16)	I (>4)
WF007104-4	R	R	R	R	R	R	I	R	I	R (>256)	R (32)	I (2)	S (1)	I (8)	I (>4)
WF007104-9	R	R	R	R	R	R	S	R	I	R (>256)	R (32)	I (2)	S (1)	R (16)	I (>4)
WF006720-3	R	R	R	R	R	R	I	R	S	R (>256)	R (32)	I (2)	I (2)	R (16)	I (8)
WF006720-4	R	R	R	R	R	R	R	R	I	R (>256)	R (32)	I (2)	I (2)	R (16)	I (8)
WF006831-5	R	R	R	I	R	R	I	R	S	R (>256)	R (32)	I (2)	I (2)	I (8)	I (8)
WF006831-6	R	R	R	R	R	R	R	R	I	R (>256)	R (32)	I (2)	I (2)	R (16)	I (8)
WF006959-1	R	R	R	S	R	R	R	R	I	R (>256)	R (32)	I (2)	I (2)	R (16)	R (16)
WF007490-3	R	R	R	R	R	R	I	R	I	R (>256)	R (>128)	I (2)	I (2)	I (8)	I (>4)
WF007490-7	R	R	R	R	R	R	R	R	I	R (>256)	R (>128)	I (2)	I (2)	R (16)	I (>4)
WF007558-1	R	R	R	I	R	R	R	R	S	R (>256)	R (>128)	I (2)	I (2)	R (16)	I (>4)
WF007766-1	R	R	R	R	R	R	R	R	I	R (>256)	R (>128)	I (2)	I (2)	R (16)	I (>4)

AMP; ampicillin, AZL; azlocillin, PEN; penicillin, AZT; aztreonam, THN; cephalothin, DNE; cephradine, URX; cefuroxime, FOX; cefoxitin, TIZ; ceftizoxime, AMX; amoxycillin, AMX/C; amoxyclavulanic acid, TAX; cefotaxime, TAX/C; cefotaxime + clavulanic acid, TAZ; ceftazidime, TAZ/C; ceftazidime + clavulanic acid. Yellow highlighting demonstrates isolates found to harbour $bla_{\text{TEM-1}}$. Breakpoints concentrations given in μg/mL

Table 8.1 shows that all 16 isolates are resistant to the 1st generation cephalosporins; cephalothin and cephradine. More variation in resistance phenotype is present with the 2nd generation cephalosporins; cefuroxime (URX) and cefoxitin (FOX). No resistance to the 3rd generation cephalosporin ceftizoxime (TIZ) is observed, in contrast to ceftazidime (TAZ) to which 10/16 are resistant. The presence of an ESBL

is indicated, but not proven by an eight-fold difference in the MIC of a 3rd generation cephalosporin and the MIC obtained when this antibiotic is used in combination with a β-lactamase inhibitor (e.g. clavulanic acid). According to these criteria, none of the isolates were thought to possess an ESBL.

Screening for bla_{TEM-1} revealed that the following isolates (isolate number is given after the sample number separated by a dash) (WF00-) 6720-3, 6720-4, 6831-5, 6831-6, 6996-3, 7104-4, 7104-9, 7490-3, 7490-7 and 7558-1 harboured this gene. These are highlighted in yellow in Table 8.1. The resistance profiles of isolates (WF00-) 6986-4, 7204-4, 7013-1, 6959-1, and 7766-1 are suggestive of AmpC-type β-lactamases. These enzymes are usually resistance to ampicillin, amoxycillin, ceftazidime (MIC > 4mg/L), cefotaxime (MIC > 1mg/L), cefoxitin (MIC > 16mg/L), and often (but not always) resistant to monobactams such as aztreonam. AmpC-type β-lactamase MICs are usually higher for ceftazidime than for cefotaxime, a result that is observed for all 16 isolates (Table 8.1).

The salmonella isolate *S.* Enteritidis 020003 was resistant to ampicillin (>128mg/L), cefaclor (>128mg/L), cefotaxime (8mg/L) and ceftazidime (>32mg/L).

8.3b Determination of the presence of an ESBL

E-test strips and double disc diffusion tests confirmed that none of the 16 commensal *E. coli* isolates harboured an ESBL. *S.* Enteritidis 020003 did have an ESBL according to these tests.

8.3c Amplification of bla_{TEM-1} *and* bla_{SHV-1} *in S.* Enteritidis *020003*

PCR with bla_{SHV-1} primers failed to detect a gene encoding an SHV-1 derivative in *S.* Enteritidis 020003. Amplification of bla_{TEM-1} in this isolate yielded a product of the expected size (858bp). The comparison of the DNA sequence of this product with

bla$_{TEM-1}$ (Lefon-Guilbout *et al.*, 2000) and *bla*$_{TEM-52}$ (Lee *et al.*, 2003; Poyart *et al.*, 1998) showed that this gene encoded *bla*$_{TEM-52}$. *Bla*$_{TEM-52}$ differs from *bla*$_{TEM-1}$ by amino acid substitutions at positions Glu-104→Lys (GAG→AAG), Met-182→Thr (ATG→ ACG) and Gly-238→ Ser (GGT→AGT). The changes identified in addition to a silent mutation identified at codon 134 are listed in Table 8.2.

Table 8.2 Comparison of *bla*$_{TEM}$ from *S.* Enteritidis 020003 and published *bla*$_{TEM-1}$, and *bla*$_{TEM-52}$ sequences

	Amino acid (codon) at position				
β–lactamase	104	134	182	238	Reference
TEM-1	Glu (GAG)	Ala (GCG)	Met (ATG)	Gly (GGT)	Leflon-Guibout *et al.*, 2000
TEM-52 (*S.* Enteritidis 02003)	Lys (AAG)	Ala (GCG)	Thr (ACG)	Ser (AGT)	This work
TEM-52 (*S.* Saint Paul)	Lys (AAG)	Ala (GCT)	Thr (ACG)	Ser (AGT)	Lee *et al.*, 2003
TEM-52 (*K. pneumoniae*)	Lys (AAG)	Ala (GCG)	Thr (ACG)	Ser (AGT)	Poyart *et al.*, 1998

8.4 Discussion

Although the 52 isolates identified in this work had reduced susceptibility to 3rd generation cephalosporins, they did not harbour any ESBLs. Similarly, Briñas *et al.* (2002) did not find any ESBLs in ampicillin resistant *E. coli* from food, humans and healthy animals. Based on susceptibility profiles, the isolates were thought to harbour either *bla*$_{TEM-1}$ (conferring high level resistance to amoxycillin), an AmpC-type β-lactamase, or both. In *E. coli*, the chromosomal *ampC* gene is normally expressed at low levels, but production of this enzyme can be increased by gene duplication or by mutation in the ampC promoter or attenuator (Caroff *et al.*, 2000; Nelson & Elisha, 1999). More recently, plasmid-mediated AmpC-type β-lactamases have also been found in *E. coli* from food-producing animals (Zhao *et al.*, 2001) and *Salmonella spp.* (Allen & Poppe, 2002).

The primary aim of this section of the chapter was to determine if the calf faecal *E. coli* harboured any ESBLs. Reduced susceptibility to the cephalosporins was demonstrated in 16 isolates, and bla_{TEM-1} was detected in ten of these. Unfortunately due to time restrictions, it was not possible to determine the exact mechanism of this reduced susceptibility in the other six isolates. The hypothesis that some of these isolates harboured AmpC β-lactamases, may be tested by isolelectric focusing and PCR to amplify and sequence the *bla*AmpC gene implicated by the pI value (Allen & Poppe, 2002; Briñas *et al.*, 2003b; Zhao *et al.*, 2001. It would also be of interest to determine if the resistance gene was transferable by conjugation, as has been found for some AmpC-type β-lactamases (Alvarez *et al.*, 2004; Knox, 1995; Zhao *et al.*, 2001).

The *S.* Enteritidis isolate was of interest because antibiotic resistance is not common in this serovar (Vahaboglu *et al.*, 2001). The isolate was implicated in a Salmonellosis outbreak during which therapeutic failures with 3rd generation cephalosporins were reported. In the treatment of extraintestinal salmonella infection the antibiotics of choice are extended-spectrum cephalosporins and fluoroquinolones. Recently, *Salmonella* isolates harbouring extended-spectrum ß-lactamases (ESBLs) capable of hydrolysing third-generation cephalosporins have been reported (Lee *et al.*, 2003; Vahaboglu *et al.*, 2001). This is of particular concern for the treatment of salmonellosis in children, because fluoroquinolones cannot be used in this age group.

The resistance detected to the 3rd generation cephalosporins ceftazidime and cefotaxime was mediated by bla_{TEM-52} (mutations at codons 104, 182 and 238, compared to bla_{TEM-1}).

A change in residue 104 from either glutamic or aspartic acid to lysine, is the second most common substitution of the TEM-type ESBLs (Knox, 1995). Here, a negatively charged residue is replaced by a positively charged residue with a long basic side chain, which may interact with the carboxylic acid group in ceftazidime, ceftibuten or aztreonam, increasing initial binding (Knox, 1995). In TEM-type ESBLs, the substitution from methionine to threonine at position 182 adds an extra hydrogen

bond between the hydroxyl group of threonine and the carbonyl group of the glutamic acid in position 64. This may increase the stability of the protein where multiple mutations are present (Poyart *et al.*, 1998). The substitution Gly-238→ Ser, occurs in 27 TEM variants and in all SHV mutants. Position 238 is in a key beta-strand of the catalytic site of class A beta-lactamases, and this mutation has been found to be critical in increasing resistance to cefotaxime (Blazquez *et al.*, 1995). A mutation at position 238 has been hypothesised to slightly expand the lower portion of the β-lactam binding site, allowing larger cephalosporins such as cefotaxime to form hydrogen bonds with residue 237 in the binding site (Huletsky *et al.*, 1993). A silent mutation was present in the $bla_{\text{TEM-52}}$ allele of isolate 020003 at position Ala-134 (GCT→ GCG) that is not present in other published salmonella TEM-52 sequences (GenBank no. AF126444, AY220520), but which has been reported in a *Klebsiella pneumoniae* TEM-52 sequence (GenBank no. Y13612).

In conclusion, reduced susceptibility to cephalosporins was found in 52/183 amp^R commensal *E. coli*, but no ESBLs were detected amongst a subset (n = 16) of these isolates. The outbreak strain *S.* Enteritidis 020003 was found to harbour an ESBL, and because antibiotic resistance is not common in this serovar the detection of an ESBL is significant. The enzyme was identified as TEM-52, which has not previously been reported in Salmonellae in the UK. The TEM-52 gene described here differed from all published salmonella TEM-52 sequences by one silent mutation.

Chapter 9. Discussion

Resistance to antibiotics arises from the use of these agents in human and animal medicine, and in animal husbandry. The advent of antibiotics into animal husbandry for treatment, but especially for prophylaxis and growth promotion, has precipitated concern that this usage poses risks to human health (Swann Committee, 1969; Salyers, 1999b; Khachatourians, 1998; van den Bogaard & Stobberingh, 2000). The debate over whether or not human health is being compromised (or will be in the future) by antibiotic use in animal husbandry is ongoing, with several working groups and independent scientists stressing the need for more surveillance of antibiotic resistance not only in pathogens but also in commensal organisms (Caprioli *et al.*, 2000; Jones *et al.*, 2000; Masterton, 2000; O'Brien, 1997). It is within this context that the key findings from this thesis are most relevant.

Chapters 3 to 8 contain detailed discussions of the specific results therein, and so this final chapter aims to address some of the wider issues emerging from these results.

To date there are many reports documenting antibiotic resistance in both human and animal pathogens, but comparatively little is known about levels of resistance in commensal organisms in food animals (Salisbury *et al.*, 2002). Notwithstanding this imbalance in the data available, the hypothesis that antibiotic uses in animals pose a risk to human health is supported by claims that commensal organisms constitute a huge reservoir of resistance genes (Salyers, 1999a; Schwarz *et al.*, 2001). On inspection of the results presented in Chapter 3, it is clear that the commensal *E. coli* of calves do harbour resistance genes to a range of antibiotics. In the unselected population these include ampicillin, amoxyclavulanic acid, cephalosporins (1st, 2nd and 3rd generation), tetracycline, streptomycin, chloramphenicol, trimethoprim and sulphamethoxazole.

The fact that isolates harbouring resistance mechanisms to these antibiotics were identified without selection to amplify their numbers indicates that they constitute a significant proportion of the population (Chapter 3). However, the relative

abundance of a particular resistance mechanism is not as important as the fact that it is present in the population. When the bacterial population is exposed to sufficient concentrations of an antibiotic, susceptible strains are suppressed and any resistant strains are amplified (Amyes *et al.*, 1996). Reduced susceptibility within the commensal *E. coli* was also found to nalidixic acid, apramycin, gentamicin and tobramycin. With the exception of apramycin, all of the antibiotics to which reduced susceptibility was demonstrated are used in human medicine. From this, the commensal *E. coli* of calves is concluded to constitute a substantial reservoir of resistance genes.

After the demonstration of numerous resistance phenotypes, the genotypes of members of the unselected and the three selected (antibiotic resistant) populations were examined. Knowledge of the background level of population diversity is essential if studies on the genetic relatedness of pathogenic or antibiotic resistant *E. coli* are to be put into context. In chapter 4, genotyping by PFGE was found to be highly discriminatory, enabling the distinction of 55 different genotypes within the commensal population, in addition to subtypes within six of these genotypes. Fewer antibiotic resistant genotypes and less diversity of genotypes correlated with increasing calf age. In the context of the current debate, these results suggest that meat from older animals is less likely to contain antibiotic resistant *E. coli* than that from calves, and so poses less of a risk to human health.

So far, the results from this research have demonstrated that the commensal *E. coli* of calves constitute a large reservoir of resistant genes, and that resistant commensal *E. coli* comprise many diverse genotypes. For human health to be compromised by the presence of these resistant commensals, meat or other foodstuff contaminated with these organisms must be ingested, or the resistance genes transferred to organisms pathogenic to humans (Piddock, 1996).

In Chapter 5, the horizontal conjugative transfer of a high frequency apramycin resistance plasmid (pUK2001) into three different genotypes was demonstrated. This transfer may have occurred *in vivo*, or in the environment before the strains colonised

the calves. Two other apramycin resistance plasmids were identified, both of which had lower transfer frequencies and were not found in multiple genotypes. Interestingly, the ability of pUK2001 to transfer into other commensal *E. coli* genotypes, isolated from the same sample as that from which pUK2001 was isolated, was demonstrated *in vitro*. Therefore, pUK2001 was capable of transfer into these genotypes, which were present in similar abundances, but had not transferred into these strains *in vivo*. This suggests that there are barriers to the spread of resistance plasmids that are present *in vivo*, but which may not be evident in *in vitro* experiments.

The nature of these barriers include the presence of restriction modification systems (Murray, 2002), the presence of other plasmids of different incompatibility groups in a potential recipient (Gordon, 1992), and spatial and temporal barriers such as the *in vivo* proximity of donors and recipients (Licht *et al.*, 1999). The application of a mathematical model to the phenomenon of plasmid transfer within a population (Chapter 6) revealed a further inhibition to the spread of plasmids. This is the growth phase of the mating pair. Experimental data of the population dynamics of plasmid transfer (chapter 6) did not fit the outcome predicted by the model. The model overestimated the numbers of transconjugants formed in the later stages of the mating experiment (from around 2.5 hours) because plasmid transfer varied with growth phase, not growth rate. In this way, the growth phase of possible donors and recipients is concluded to be a potential barrier to plasmid spread in a mixed population.

The results of chapter 6 also demonstrate that the dynamics of plasmid transfer cannot be simply explained in terms of cell densities, growth rates and plasmid transfer rates. The model predicts that plasmids with high transfer rates will be present at a greater prevalence than plasmids with lower transfer rates. These comparative prevalences are not observed *in vivo*, demonstrating that many more factors influence the dynamics of plasmid transfer than may be incorporated into an *in vitro* experiment (see chapter 6 for details).

A fitness advantage was observed in *E. coli* hosts harbouring pUK2001 or pUK2002, compared to their isogenic counterparts. This is the first demonstration of an *in vitro* fitness advantage of plasmids obtained from commensal isolates. The fitness advantages measured were comparable to fitness advantages described by other studies of cloning vectors and common laboratory plasmids. However, the fact that these plasmids required selection in order to be isolated from the commensal *E. coli* population shows that within this population they are rare, and so the *in vivo* advantage must be slight, if present at all.

The results from chapters 6 and 7 are very interesting in themselves, but their full interpretation requires a comparison with the situation *in vivo*. This comparison phase has been omitted in some earlier studies (Dahlberg & Chao, 2003; Lenski *et al.*, 1994; Modi *et al.*, 1992). The results of such *in vitro* experiments have been used to support the hypothesis that resistant bacteria in food animals are potential risks to human health (McGeer, 1998; Salyers & Amabile-Cuevas, 1997). Chapters 6 and 7 demonstrate that although apramycin resistance plasmids may transfer horizontally to other *E. coli*, and may not confer a competitive disadvantage on the host bacterium, *in vivo*, there are barriers to the spread of these plasmids. The low prevalences of these plasmids suggest that the cost of carriage is also likely to be greater *in vivo*.

As mentioned earlier in this discussion, one potential hazard posed by the presence of resistant bacteria in the flora of food animals is the contamination of foodstuff for human consumption. This is most easily tracked with zoonotic organisms such as *Salmonella* spp. Salmonella is a common resident of the calf intestinal flora, and causes infections and outbreaks in humans following ingestion (Threlfall, 2002). One such outbreak is described in chapter 8. Here the mechanism of resistance was found to be an ESBL. To the best of my knowledge ESBLs have not yet been found within the commensal organisms of calves, and were not harboured by the *E. coli* isolates characterised in this thesis. Based on this evidence, and the fact that cephalosporins are commonly used in the treatment of extraintestinal salmonella infections and other serious infections (Amyes *et al.*, 1996), the most likely way in which the salmonella

strain causing the outbreak acquired its plasmid-mediated resistance was from human usage of cephalosporins.

There are a few reports where resistance mechanisms present in human isolates are thought to have been acquired from animal isolates (Fey *et al.*, 2000; Spika *et al.*, 1987; van den Bogaard *et al.*, 1997), but the majority of resistance in human isolates does not appear to have arisen from the use of antibiotics in animals, but from their use in human medicine (Cook, 1997; Phillips *et al.*, 2004). Although antibiotic resistant bacteria are present within the flora of food animals, the way to reduce the potential hazard this poses is to minimise the spread of organisms between animals and humans. This can be achieved by better food hygiene practices, such as reducing cross contamination of raw animal products and cooked foods, washing fruit and vegetables (as manure is often used in their cultivation), and adequate cooking of meat based products.

In conclusion, the work presented in this thesis demonstrates that commensal *E. coli* of calves do represent a substantial reservoir of resistance genes, of which resistance to apramycin has been demonstrated to have spread horizontally. For resistant organisms to pose a threat to human health, they must either contaminate foodstuffs or transfer their resistance mechanisms to pathogenic organisms. Although the transfer of apramycin resistance plasmids has been demonstrated, *in vivo* barriers exist that are capable of preventing extensive spread throughout a population. The risk posed by the contamination of foodstuffs can be minimised by good food hygiene practices. From the evidence provided by this thesis, it is suggested that the use of antibiotics in animal husbandry poses far less of a risk to human health than the use of these agents in human medicine. All antibiotic usage provides a selective pressure for the emergence of resistance, and efforts now need to be concentrated on reducing antibiotic usage and keeping the reservoirs of animal and human resistant organisms separate.

References cited

Aarestrup, F.M., Kruse, H., Tast, E., Hammerum, A.M., and Jensen, L.B. (2000). Associations between the use of antimicrobial agents for growth promotion and the occurrence of resistance among *Enterococcus faecium* from broilers and pigs in Denmark, Finland, and Norway. *Microbial Drug Resistance* **6**, 63-70.

Abraham, E.P., and Chain, E. (1940). An enzyme from bacteria able to destroy penicillin. *Nature* **146**, 837.

Allen, K.J., and Poppe, C. (2002). Occurrence and characterisation of resistance to extended-spectrum cephalosporins mediated by beta-lactamase CMY-2 in *Salmonella* isolated from food-producing animals in Canada. *Canadian Journal of Veterinary Research* **66**, 137-144.

Alvarez, M., Tran, J.H., Chow, N., and Jacoby, G.A. (2004). Epidemiology of conjugative plasmid-mediated AmpC beta-lactamases in the United States. *Antimicrobial Agents and Chemotherapy* **48**, 533-537.

Amyes, S.G.B. and Smith, J.T. (1974). R-factor trimethoprim resistance mechanism: an insusceptible target site. *Biochemical and Biophysical Research Communications* **58**, 412-418.

Amyes, S.G.B., Thomson, C.J., Miles, R.S., and Tillotson, G. (1996). Antimicrobial chemotherapy. Publishers: Martin Dunitz, London, UK.

Anadon, A., Martinez-Larranga, M.R., Diaz, M. J., Fernandez-Cruz, M.L., Martinez, M.A., Frejo, M.T., Martinez, M., Fernandez, R., Anton, R.M., Morales, M.E., and Tafur, M. (1999). Pharmacokinetic variables and tissue residues of enrofloxacin and ciprofloxacin in healthy pigs. *American Journal of Veterinary Research* **60**, 1377-1382.

Andrews, J.H., and Hall, R.F. (1986). *r*- and *K* selection and microbial ecology. *Advances in Microbial Ecology* **9**, 99-147.

Andrews, J.M. (2001). Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy* **48**, Supplement S1, 5-16.

Andrews, J.M., Walker, R., and King, A. (2002). Evaluation of media for testing the susceptibility of *Pseudomonas aeruginosa* by BSAC methodology. *Journal of Antimicrobial Chemotherapy* **50**, 479-486.

Andrup, L. and Anderson, K. (1999). A comparison of the kinetics of plasmid transfer in the conjugative systems encoded by the F plasmid from *Escherichia coli* and plasmid pCF10 from *Enterococcus faecalis*. *Microbiology* **145**, 2001-2009.

Antunes, P., Machado, J., Sousa, J.C., and Peixe, L. (2004). Dissemination amongst humans and food products of animal origin of a *Salmonella typhimurium* clone expressing an integron-borne OXA-30 beta-lactamase. *Journal of Antimicrobial Chemotherapy* **54**, 429-434.

Arana, I.I., Justo, J.I., Muela, A., Pocino, M., Iriberri, J., and Barcina, I.I. (1997). Influence of a survival process in a freshwater system upon plasmid transfer between *Escherichia coli* strains. *Microbial Ecology* **33**, 41-49.

Arcangioli, M.A., Leroy-Setrin, S., Martel, J.L., and Chaslus-Dancla, E. (1999). A new chloramphenicol and florfenicol resistance gene flanked by two integron structures in *Salmonella typhimurium* DT104. *FEMS Microbiology Letters* **174**, 327-332.

Aslam, M., Nattress, F., Greer, G., Yost, C., Gill, C. and McMullen, L. (2003). Origin of contamination and genetic diversity of *Escherichia coli* in beef cattle. *Applied and Environmental Microbiology* **69**, 2794-2799.

Atlas, R.M. and Bartha, R. (1998). Development of microbial communities. In: *Microbial Ecology - fundamentals and applications.* Addison Wesley Longman, California. Chapter 6, p. 174-217.

Avelar, K.E., Pinto, L.J., Antunes, L.C., Lobo, L.A., Bastos, M.C., Domingues, R.M., and Ferreira, M.C. (1999). Production of bacteriocin by *Bacteriodes fragilis* and partial characterization. *Letters in Applied Microbiology* **29**, 264-268.

Bachmann, B. (1972). Pedegrees of some mutant strains of *Escherichia coli* K-12. *Bacteriological Reviews* **36**, 525-557.

Bartie, K.L., Wilson, K.J., Williams, D.W., and Lewis. M.A.O. (2000). Macrorestriction fingerprinting of "*Streptococcus milleri*" group bacteria by pulsed-field gel electrophoresis. *Journal of Clinical Microbiology* **38**, 2141-2149.

Bass, L., Liebert, C.A., Lee, M.D., Summers, A.O., White, D.G., Thayer, S.G., and Maurer, J.J. (1999). The incidence and characterization of integrons, genetic elements associated with multiple drug resistance, in avian *Escherichia coli. Amtimicrobial Agents and Chemotherapy* **43**, 2925-2929.

Bates, J., Jordens, J.Z., and Griffiths, D.T. (1994). Farm animals as a putative reservoir for vancomycon-resistant enterococcal infection in man. *Journal of Antimicrobial Chemotherapy* **34**, 507-516.

Benveniste, R., and Davies, J. (1971). Enzymatic acetylation of aminoglycoside antibiotics by *Escherichia coli* carrying an R factor. *Biochemistry* **10**, 1787-1796.

Bergeron, M.G. and Ouellette, M. (1998). Preventing antibiotic resistance through rapid genotypic identification of bacteria and of their antibiotic resistance genes in the clinical microbiology laboratory. *Journal of Clinical Microbiology* **36**, 2169-2172.

Bjorkman, J., Hughes, D., and Andersson, D. (1998). Virulence of antibiotic-resistant *Salmonella typhimurium. Proceedings of the National Academy of Science USA* **95**, 3949-3953.

Bjorkman, J., and Andersson, D.I. (2000). The cost of antibiotic resistance from a bacterial perspective. *Drug Resistance Updates* **3**, 237-245.

Blazquez, J., Morosini, M.I., Negri, M.C., Gonzalez-Leiza, M., and Baquero, F. (1995). Single amino acid replacements at positions altered in naturally occurring extended-spectrum TEM beta-lactamases. *Antimicrobial Agents and Chemotherapy* **39**, 145-149.

Block, C., Davidson, Y., and Keller, N. (1998). Unreliability of disc diffusion test for screening for reduced penicillin susceptibility in *Neisseria meningitidis. Journal of Clinical Microbiology* **36**, 3103-3104.

Boerlin, P., Wissing, A., Aarestrup, F.M., Frey, J., and Nicolet, J. (2001). Antimicrobial growth promoter ban and resistance to macrolides and vancomycin in *enterococci* from pigs. *Journal of Clinical Microbiology* **39**, 4193-4195.

Bohannan, B.J.M., and Hughes, J. (2003). New approaches to analysing microbial biodiversity data. *Current Opinion in Microbiology* **6**, 282-287.

Bouma, J.E., and Lenski, R.E. (1988). Evolution of a bacteria/plasmid association. *Nature* **335**, 351-352.

Bradford, P.A., Cherubin, C.E., Idemyor, V., Rasmussen, B.A., and Bush, K. (1994). Multiply resistant *Klebsiella pneumoniae* strains from two Chicago hospitals: identification of the extended-spectrum TEM-12 and TEM-10 ceftazidime-hydrolysing β-lactamases in a single isolate. *Antimicrobial Agents and Chemotherapy* **38**, 761-766.

Bradford, P.A. (2001). Extended-spectrum β-lactamases in the 21st century: Characterization, epidemiology, and detection of this important resistance threat. *Clinical Microbiology Reviews* **14**, 933-951.

Bräu, B., Pilz, U., and Piepersberg, W. (1984). Genes for gentamicin-(3)-N-acetyltransferases III and IV: I. Nucleotide sequence of the AAC(3)-IV gene and possible involvement of an IS140 element in its expression. *Molecular and General Genetics* **193**, 179-187.

Briggs, C.E., and Fratamico, P.M. (1999). Molecular characterization of an antibiotic resistance gene cluster of *Salmonella typhimurium* DT104. *Antimicrobial Agents and Chemotherapy* **43**, 846-849.

Briñas, L., Zarazaga, M., Sáenz, Y., Ruiz-Larrea, F., and Torres, C. (2002). β - lactamases in ampicillin-resistant *Escherichia coli* isolates from foods, humans, and healthy animals. *Antimicrobial Agents and Chemotherapy* **46**, 3156-3163.

Briñas, L., Moreno, M.A., Teshager, T., Zarazaga, M., Sáenz, Y., Porrero, C., Dominguez, L., and Torres, C. (2003a). Beta-lactamase characterisation in *Escherichia coli* isolates with diminished susceptibility or resistance to extended-spectrum cephalosporins recovered from sick animals in Spain. *Microbial Drug Resistance* **9**, 201-209.

Briñas, L., Moreno, M.A., Zarazaga, M., Porreor, C., Sáenz, Y., García, M., Dominguez, L., and Torres, C. (2003b). Detection of CMY-2, CTX-M-14, and SHV-12 β-lactamases in *Escherichia coli* faecal isolates from healthy chickens. *Antimicrobial Agents and Chemotherapy* **47**, 2056-2058.

Bronzwaer, S., Buchholz, U., Courvalin, P., Snell, J., Cornaglia, G., de Neeling, A., Aubry-Damon, H., Degener, J., and EARSS participants. (2002). Comparability of antimicrobial susceptibility tests from 22 European countries and Israel: An external quality assurance exercise of the European Antimicrobial Resistance Surveillance System (EARSS) in collaboration with the United Kingdom National External Quality Assurance Scheme (UK NEQA). *Journal of Antimicrobial Chemotherapy* **50**, 953-964.

Brown, A.M.C., and Willetts, N.S. (1981). A physical and genetic map of the IncN plasmid R46. *Plasmid* **5**, 188-201.

BSAC (2004). Disc diffusion method for antimicrobial susceptibility testing. http://www.bsac.org.uk/uploads/jan%202004%20website%20version%203.pdf. Date last accessed; 19th July 2004.

Burman, L.G., Nordstrom, K., and Bloom, G.D. (1972). Murein and the outer penetration barrier of *Escherichia coli* K-12, *Proteus mirabilis*, and *Pseudomonas aeruginosa*. *Journal of Bacteriology* **112**, 1364-1374.

Bush, K., Jacoby, G.A., Medeiros, A.A. (1995). A functional classification scheme for beta-lactamases and its correlation with molecular structure. *Antimicrobial Agents and Chemotherapy* **39**, 1211-1233.

Calderwood, S.B., Gardella, A., Philippon, A.M., Jacoby, G.A., and Moellering, R.C.Jr. (1982). Effects of azlocillin in combination with clavulanic acid, sulbactam, and N-formimidoyl thienamycin against beta-lactamase-producing, carbenicillin-resistant *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **22**, 266-271.

Caprioli, A., Busani, L., Martel, J.L., and Helmuth, R. (2000). Monitoring of antibiotic resistance in bacteria of animal origin: epidemiological and microbiological methodologies. *International Journal of Antimicrobial Agents* **14**, 295-301.

Carlson, C.A., Pierson, L.S., Rosen, J.J., and Ingraham, J.L. (1983). *Pseudomonas stutzeri* and related species undergo natural transformation. *Journal of Bacteriology* **153**, 93-99.

Caroff, N., Espaze, E., Gautreau, D., Richet, H. and Reynaud, A. (2000). Analysis of the effects of –42 and –32 *ampC* promoter mutations in clinical isolates of *Escherichia coli* hyperproducing AmpC. *Journal of Antimicrobial Chemotherapy* **33**, 783-788.

Casewell, M., Friis, C., Marco, E., McMullin, P., and Phillips, I. (2003). The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *Journal of Antimicrobial Chemotherapy* **52**, 159-161.

Catlin, B.W. (1960). Transformation of *Neisseria meningitidis* by deoxyribonucleates from cells and from culture slime. *Journal of Bacteriology* **79**, 579-590.

Caugant, D.A., Levin, B.R., and Selander, R.K. (1981). Genetic diversity and temporal variation in the *E. coli* population of a human host. *Genetics* **98**, 467-490.

Chadwick, D.J., and Goode, J. (1997). Antibiotic resistance: origins, evolution, selection, and spread. *Ciba Foundation Symposium* **207**, 1-3.

Chanal, C., Bonnet, R., De Champs, C,, Sirot, D., Labia, R., and Sirot, J. (2000). Prevalence of beta-lactamases among 1,072 clinical strains of *Proteus mirabilis*: a 2-year survey in a French hospital. *Antimicrobial Agents and Chemotherapy* **44**, 1930-1935.

Chao, A. (1987). Estimating the population size for capture-recapture data with unequal catchability. *Biometrics* **43**, 783-791.

Chaslus-Dancla, E., Martel, J.L., Carlier, C., Lafont, J.P., and Courvalin, P. (1986). Emergence of aminoglycoside 3-N-acetyltransferase IV in *Escherichia coli* and *Salmonella typhimurium* isolated from animals in France. *Antimicrobial Agents and Chemotherapy* **29**, 239-243.

Chaslus-Dancla, E., Glupcznski, Y., Gerbaud, G., Lagorce, M., Lafont, J.P., and Courvalin, P. (1989). Detection of apramycin resistant Enterobacteriaceae in hospital isolates. *FEMS Microbiology Letters* **61**, 261-266.

Chaslus-Dancla, E., Pohl, P., Meurisse, M., Marin, M., Lafont, J.P. (1991). High genetic homology between plasmids of human and animal origin conferring resistance to the aminoglycosides gentamicin and apramycin. *Antimicrobial Agents and Chemotherapy* **35**, 590-593.

Chen, H.Y., Yuan, M., Ibrahim-Elmagboul, I.B., Livermore, D.M. (1995). National survey of susceptibility to antimicrobials amongst clinical isolates of *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy* **35**, 521-534.

Cohen, S.P., Hooper, D.C., Wolfson, J.S., Souza, K.S., McMurry, L.M., Levy, S.B. (1988). Endogenous active efflux of norfloxacin in susceptible *Escherichia coli. Antimicrobial Agents and Chemotherapy* **32**, 1187-1191.

Cohen, S.P., Hachler, H., and Levy, S.B. (1993). Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli. Journal of Bacteriology* **175**, 1484-1492.

Collis, C.M, Grammaticopoulos, G., Briton, J., Stokes, H.W., and Hall, R.M. (1993). Site-specific insertion of gene cassettes into integrons. *Molecular Microbiology* **9**, 41-52.

Colwell, R.K., and Coddington, J.A. (1994). Estimating terrestrial biodiversity through extrapolation. *Philosophical Transactions of the Royal Society (London) series B* **345**, 101-118.

Cook, R.R. (1997). Antimicrobial resistance – use in veterinary medicine and human medicine. *Journal of Antimicrobial Agents and Chemotherapy* **39**, 435.

Cooper, A.J., Shoemaker, N.B., and Salyers, A.A. (1996). Erythromycin resistance gene from the *Bacteroides* conjugal transposon Tc-r Em(r) 7853 is nearly identical to *ermG* from *Bacillus sphaericus. Antimicrobial Agents and Chemotherapy* **40**, 506-508.

Corpet, D.E (1987). Antibiotic residues and drug resistance in human intestinal flora. *Antimicrobial Agents and Chemotherapy* **31**, 587-593.

Cox, E.C., White, J.R., Flaks, J.G. (1964). Streptomycin action and the ribosome. *Proceedings of the National Academy of Sciences USA* **51**, 703-709.

Crawley, M.J. (2002). Statistical computing: an introduction to data analysis using S-Plus. John Wiley & Sons, Chichester.

Cullum, J., Collins, J.F., and Broda, P. (1978). Factors affecting the kinetics of progeny formation with F'*lac* in *Escherichia coli* K12. *Plasmid* **1**, 536-544.

Curtiss, R., Caro, L.G., Allison, D.P. and Stallions, D.R. (1969). Early stages of conjugation in *Escherichia coli. Journal of Bacteriology* **100**, 1091-1104.

Dahlberg, C., and Chao, L. (2003). Amelioration of the cost of conjugative plasmid carriage in *Escherichia coli* K12. *Genetics* **165**, 1641-1649.

Danel, F., Hall, L.M., Duke, B., Gur, D., and Livermore, D.M. (1999). OXA-17, a further extended-spectrum variant of OXA-10 beta-lactamase, isolated from *Pseudomonas aeruginosa. Antimicrobial Agents and Chemotherapy* **43**, 1362-1366.

DANMAP (1998). Consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. Statens Serum Institut, Danish Veterinary and Food Administration, Danish Medicines Agency, Danish Veterinary Laboratory, Copenhagen, Denmark. ISSN 1397-1409.

DANMAP (2002). Consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. Statens Serum Institut, Danish Veterinary and Food Administration, Danish Medicines Agency, Danish Veterinary Laboratory, Copenhagen, Denmark. ISSN 1600-2032.

Darville, J.M., and Lovering, A.M. (2003). Variation in the content of ciprofloxacin susceptibility testing discs: Its effect on the BSAC disc method. *Journal of Antimicrobial Chemotherapy* **51**, 465-467.

Datta, N., and Kontomichalou, P. (1965). Penicillinase sysnthesis controlled by infectious R factors in Enterobacteriacae. *Nature* **208**, 239-244.

Datta, N., Hedges, R.W., Shaw, E.J., Sykes, R.B., and Richmond, M.H. (1971). Properties of an R factor from *Pseudomonas aeruginosa*. *Journal of Bacteriology* **108**, 1244-1249.

Davies, J., and O'Connor, S. (1978). Enzymatic modification of aminoglycoside antibiotics: 3-N-aminoacetyltransferase with broad specificity that determines resistance to the novel aminoglycoside apramycin. *Antimicrobial Agents and Chemotherapy* **14**, 69-72.

Davies, J., and Webb, V. (1998). Antibiotic resistance in bacteria. In: *Emerging Infections*, Academic Press, pages 239-273.

De Champs, C., Sirot, D., Chanal, C., Bonnet, R., Sirot, J., and the French Study Group. (2000). A 1998 survey of extended-spectrum β-lactamases in *Enterobacteriacae* in France. *Antimicrobial Agents and Chemotherapy* **44**, 3177-3179.

Delsol, A.A., Anjum, M., Woodward, M.J., Sunderland, .J, and Roe, J.M. (2003). The effect of chlortetracycline treatment and its subsequent withdrawal on multi-resistant *Salmonella enterica* serovar Typhimurium DT104 and commensal *Escherichia coli* in the pig. *Journal of Applied Microbiology* **95**, 1226-1234.

Dencer, H.G., Slaney, L., Maclean, I.W., and Albritton, W.L. (1982). Mobilization of nonconjugative antibiotic resistance plasmids in *Haemophilus ducreyi*. *Journal of Bacteriology* **149**, 726-732.

Diez-Gonzalez, F., Calloway, T.R., Kizoulis, M.G., and Russell, J.B. (1998). Grain feeding and dissemination of acid-resistant *Escherichia coli* from cattle. *Science* **281**, 1666-1668.

Dijkshoorn, L., and Towner, K. (2001). An introduction to the generation and analysis of microbial typing data. In: New Approaches for the Generation and Analysis of Microbial Typing Data. Editors; Dijkshoorn, L., Towner, K., and Struelens, M. Elsevier Science, Amsterdam, The Netherlands.

Donabedian, S., Thal, L.A., Bozigar, P., Zervos, T., Hershberger, E., and Zervos, M. (2003a). Antimicrobial resistance in swine and chickens fed virginiamycin for growth promotion. *Journal of Microbiological Methods* **55**, 739-743.

Donabedian, S.M., Thal, L.A., Hershberger, E., Perri, M.B., Chow, J.W., Bartlett, P., Jones, R., Joyce, K., Rossiter, S., Gay, K., Johnson, J., Mackinson, C., Debess, E., Madden, J., Angula, F., and Zervos, M.J. (2003b). Molecular characterization of gentamicin-resistant *Enterococci* in the United States: Evidence of spread from animals to humans through food. *Journal of Clinical Microbiology* **41**, 1109-1113.

Doucet-Populaire, F., Trieucuot, P., Andremont, A., and Courvalin, P. (1992). Conjugal transfer of plasmid DNA from *Enterococcus-faecalis* to *Escherichia-coli* in digestive tracts of gnotobiotic mice. *Antimicrobial Agents and Chemotherapy* **36**, 502-504.

Dowson, C.G., Hutchison, A., Woodford, N., Johnson, A.P., George, R.C., and Spratt, B.G. (1990). Penicillin-resistant viridans *Streptococci* have obtained altered penicillin-binding protein genes from penicillin- resistant strains of *Streptococcus-pneumoniae*. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 5858-5862.

Dubnau, D. (1991). The regulation of genetic competence in *Bacillus-subtilis*. *Molecular Microbiology* **5**, 11-18.

Dunbar, J., Takala, S., Barns, S.M., Davis, J.A., and Kuske, C.R. (1999). Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. *Applied and Environmental Microbiology* **65**, 1662-1669.

Dunlop, R.H., McEwen, S.A., Meek, A.H., Clarke, R.C., Black, W.D., and Friendship, R.M. (1998). Associations among antimicrobial drug treatments and antimicrobial resistance of fecal *Escherichia coli* of swine on 34 farrow-to-finish farms in Ontario, Canada. *Preventative Veterinary Medicine* **34**, 283-305.

Edelstein, M., Pimkin, M., Dmitrachenko, T., Semenov, V., Kozlova, N., Gladin, D., Baraniak, A., and Stratchounski, L. (2004). Multiple outbreaks of nosocomial Salmonellosis in Russia and Belarus caused by a single clone of *Salmonella enterica* Serovar Typhimurium producing an extended-spectrum beta-lactamase. *Antimicrobial Agents and Chemotherapy* **48**, 2808-2815.

Edgar, R., and Bibi, E. (1997). MdfA, an *Escherichia coli* multidrug resistance protein with an extraordinarily broad spectrum of drug recognition. *Journal of Bacteriology* **179**, 2274-2280.

Edwards, G.F.S. (2003). Personal communication.

Eisenstein, B.I. (1990). New molecular techniques for microbial epidemiology and the diagnosis of infectious diseases. *The Journal of Infectious Diseases* **161**, 595-602.

Emborg, H.D., Andersen, J.S., Seyfarth, A.M., Andersen, S.R., Boel, J., and Wegener, H.C. (2003). Relations between the occurrence of resistance to antimicrobial growth promoters among *Enterococcus faecium* isolated from broilers and broiler meat. *International Journal of Food Microbiology* **84**, 273-284.

Endtz, H.P., Ruijs, G.J., Vanklingeren, B., Jansen, W.H., van der Reyden, T., and Mouton, R.P. (1991). Quinolone resistance in *Campylobacter* isolated from man and poultry following the introduction of fluoroquinolones in veterinary-medicine. *Journal of Antimicrobial Chemotherapy* **27**, 199-208.

Enne, V.I., Delsol, A.A., Roe, J.M., and Bennett, P.M. (2004a). Rifampicin resistance and its fitness cost in *Enterococcus faecium. Journal of Antimicrobial Chemotherapy* **53**, 203-207.

Enne, V.I., Bennet, P.M., Livermore, D.M., and Hall, L.M.C. (2004b). Enhancement of host fitness by the *sul2*-coding plasmid p9123 in the absence of selective pressure. *Journal of Antimicrobial Chemotherapy* **53**, 958-963.

Everett, M.J., Jin, Y.F., Ricci, V., and Piddock, L.J. (1996). Contributions of individual mechanisms to fluoroquinolone resistance in 36 *Escherichia coli* strains isolated from humans and animals. *Antimicrobial Agents and Chemotherapy* **40**, 2380-2386.

Everitt, R.S. (1989). *Statistical Methods for Medical Investigations*. Oxford University Press, New York/Edward Arnold, London.

Feighner, S.D., and Dashkevicz, M.P. (1987). Subtherapeutic levels of antibiotics in poultry feeds and their effects on weight gain, feed efficiency, and bacterial cholyltaurine hydrolase activity. *Applied and Environmental Microbiology* **53**, 331-336.

Fermer, C., Kristiansen, B.E., Skold, O., Swedberg, G. (1995). Sulfonamide resistance in *Neisseria meningitidis* as defined by site-directed mutagenesis could have its origin in other species. *Journal of Bacteriology* **177**, 4669-4675.

Fernandez-Astorga, A., Muela, A., Cisterna, R., Iriberri, J., and Barcina, I. (1992). Biotic and abiotic factors affecting plasmid transfer in *Escherichia coli* strains. *Applied and Environmental Microbiology* **58**, 392-398.

Fey, P.D., Safranek, T.J., Rupp, M.E., Dunne, E.F., Ribot, E., Iwen, P.C., Bradford, P.A., Angulo, F.J., and Hinrichs, S.H. (2000). Ceftriaxone-resistant salmonella infection acquired by a child from cattle. *The New England Journal of Medicine* **342**, 1242-1249.

Fischer, W., and Haas, R. (2004). The RecA protein of *Helicobacter pylori* requires a posttranslational modification for full activity. *Journal of Bacteriology* **186**, 777-784.

Foxman, B., Manning, S.D., Tallman, P., Bauer, R., Zhang, L., Koopman, J.S., Gillespie, B., Sobel, J.D., and Marrs, C.F. (2002). Uropathogenic *Escherichia coli* are more likely than commensal *E. coli* to be shared between heterosexual sex partners. *American Journal of Epidemiology* **156**, 1133-1140.

Franke, A.E., and Clewell, D.B. (1981). Evidence for a chromosome-borne resistance transposon (TN916) in *Streptococcus faecalis* that is capable of conjugal transfer in the absence of a conjugative plasmid. *Journal of Bacteriology* **145**, 494-502.

Freter, R. (1981). Mechanism of association of bacteria with mucosal surfaces. *Ciba Symposium Foundation* **80**, 36-47.

Freter, R., Brickner, H., Fekete, J., Vickerman, M.M., and Carey, K.E. (1983). Survival and implantation of *Escherichia coli* in the intestinal tract. *Infection and Immunity* **39**, 686-703.

Frischer, M.E., Thurmond, J.M., and Paul, J.H. (1990). Natural plasmid transformation in a high-frequency-of-transformation marine *Vibrio* strain. *Applied and Environmental Microbiology* **56**, 3439-3444.

Gaskins, H.R., Collier, C.T., and Anderson, D.B. (2002). Antibiotics as growth promotants: mode of action. *Animal Biotechnology* **13**, 29-42.

Gautom, R.K. (1997). Rapid pulsed-field gel electrophoresis protocol for typing of *Escherichia coli* O157:H7 and other gram-negative organisms in 1 day. *Journal of Clinical Microbiology* **35**, 2977-2980.

Gemmell, C.G. (1999). Where is typing going? *Journal of Hospital Infection* **43**, (Supplement) S89-S92.

Gillespie, S.H. (2001). Antibiotic resistance in the absence of selective pressure. *International Journal of Antimicrobial Agents* **17**, 171-176.

Goldberg, I.D., Gwinn, D.D., and Thorne, C.B. (1966). Interspecies transformation between *Bacillus subtilis* and *Bacillus licheniformis*. *Biochemical and Biophysical Research Communications* **23**, 543-548.

Goodwin, D., and Slater, J.H. (1979). The influence of the growth environment on the stability of a drug resistant plasmid in *Escherichia coli* K12. *Journal of General Microbiology* **111**, 201-210.

Gordon, D.M. (1992). Rate of plasmid transfer among *Escherichia coli* strains isolated from natural populations. *Journal of General Microbiology* **138**, 17-21.

Gormley, E.P., and Davies, J. (1991). Transfer of plasmid RSF1010 by conjugation from *Escherichia coli* to *Streptomyces lividans* and *Mycobacterium smegmatis*. *Journal of Bacteriology* **173**, 6705-6708.

Goze, A., and Ehrlich, S.D. (1980). Replication of plasmids from *Staphhylococcus aureus* in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 7333-7337.

Grant, A.J., and Pittard, J. (1974). Incompatibility reactions of R-plasmids isolated from *Escherichia coli* of animal origin. *Journal of Bacteriology* **120**, 185-190.

Gulay, Z., Biemen, M., Amyes, S.G.B., and Yulug, N. (2000). Beta-lactamase patterns and betalactam/clavulanic acid resistance in *Escherichia coli* isolated from fecal samples from healthy volunteers. *Journal of Chemotherapy* **12**, 208-215.

Gunn, G.J., and Low, J.C. (2003). Analysis of passive surveillance data for antimicrobial resistance from cases of neonatal bovine enteritis. *Veterinary Record* **152**, 537-539.

Haas, C.E., Nix, D.E., and Schentag, J.J. (1990). *In vitro* selection of resistant *Helicobacter-pylori*. *Antimicrobial Agents and Chemotherapy* **34**, 1637-1641.

Hahm, B.K., Maldonado, Y., Schreiber, E, Bhunia, A.K. and Nakatsu, C.H. (2003). Subtyping of foodborne and environmental isolates of *Escherichia coli* by multiplex-PCR, rep-PCR, PFGE, ribotyping and AFLP. *Journal of Microbiological Methods* **53**, 387-399.

Hall, L.M., Livermore, D.M., Gur, D., Akova, M., and Akalin, H.E. (1993). OXA-11, an extended-spectrum variant of OXA-10 (PSE-2) beta-lactamase from *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **37**, 1637-1644.

Hardy, K. (1984). *Bacterial plasmids. Aspects of Microbiology*. Van Nostrand Reinhold (UK) Co. Ltd, Wokingham, Berkshire, England.

Hartl, D.L., Dykhuizen, D.E., Miller, R.D., Green, L., and de Framond, J. (1983). Transposable element IS50 improves growth rates of *E. coli* cells without tranposition. *Cell* **35**, 503-510.

Hartl, D.L., and Dykhuizen, D.E. (1984). The population genetics of *Escherichia coli*. *Annual Reviews in Genetics* **18**, 31-68.

Hartman, A.B., Essiet, I.I., Isenbarger, D.W. and Lindler, L.E. (2003). Epidemiology of tetracycline resistance determinants in Shigella *spp.* and enteroinvasive *Escherichia coli*: characterization and dissemination of tet(A)-1. *Journal of Clinical Microbiology* **41**, 1023-1032.

Heffron, F., Sublett, R., Hedges, R.W., Jacob, A., and Falkow, S. (1975). Origin of the TEM-beta-lactamase gene found on plasmids. *Journal of Bacteriology* **122**, 250-256.

Heinzel, P., Werbitzky, O., Distler, J., and Piepersberg, W. (1988). A second streptomycin resistance gene from *Streptomyces griseus* codes for streptomycin-3"-phosphotransferase. Relationships between antibiotic and protein kinases. *Archives of Microbiology* **150**, 184-92.

Heir, E., Lindstedt, B.A., Leegaard, T.M., Gjernes, E., and Kapperud, G. (2004). Prevalence and characterization of integrons in blood culture Enterobacteriaceae and gastrointestinal *Escherichia coli* in Norway and reporting of a novel class 1 integron-located lincosamide resistance gene. *Annals of Clinical Microbiology and Antimicrobials* **3**, 12.

Helling, R.B., Kinney, T., and Adams, J. (1981). The maintenance of plasmid-containing organisms in populations of *Escherichia coli*. *Journal of General Microbiology* **123**, 129-141.

Heritage, J., M'Zali, F.H., Gascoyne-Binzi, D., Hawkey, P.M. (1999). Evolution and spread of SHV extended-spectrum beta-lactamases in gram-negative bacteria. *Journal of Antimicrobial Chemotherapy* **44**, 309-318.

Hinton, M., Rixson P.D., Allen V., and Linton A.H. (1984). The persistence of drug resistant *Escherichia coli* in the majority faecal flora of calves. *Journal of Hygiene (London)* **93**, 547-557.

Hinton, M., Linton, A.H., and Hedges, A.J. (1985). The ecology of *Escherichia coli* in calves reared as dairy-cow replacements. *Journal of Applied Bacteriology* **58**, 131-138.

Hinton, M. (1986). The ecology of *Escherichia coli* in animals including man with particular reference to drug resistance. *Veterinary Record* **119**, 420-426.

Hirai K., Aoyama, H., Irikura, T., Iyobe, S., and Mitsuhashi S. (1986a). Differences in susceptibility to quinolones of outer membrane mutants of *Salmonella typhimurium* and *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* **29**, 535-538.

Hirai, K., Aoyama, H, Suzue, S., Irikura, T., Iyobe, S., and Mitsuhashi, S. (1986b). Isolation and characterization of norfloxacin-resistant mutants of *E. coli* K12. *Antimicrobial Agents and Chemotherapy* **30**, 248-253.

Hollingshead, S., and Vapnek, D. (1985). Nucleotide sequence analysis of a gene encoding a streptomycin/spectinomycin adenylyltransferase. *Plasmid* **13**, 17-30.

Hovde, C.J., Austin, P.R., Cloud, K.A., Williams, C.J., and Hunt, C.W. (1999). Effect of cattle diet on *Escherichia coli* O157:H7 acid resistance. *Applied and Environmental Microbiology* **65**, 3233-3235.

Howe, K., and Linton, A.H. (1976a). The distribution of O-antigen types *of Escherichia coli* in normal calves, compared with man, and their R plasmid carriage. *Journal of Applied Bacteriology* **40**, 317-330.

Howe, K., Linton, A.H., and Osborne, A.D. (1976b). A longitudinal study of *Escherichia-coli* in cows and calves with special reference to the distribution of O-antigen types and antibiotic resistance. *Journal of Applied Bacteriology* **40**, 331-340.

Hoyle, D.V., Knight, H.I., Shaw, D.J., Hillman, K., Pearce, M.C., Low, J.C., Gunn, G.J., Woolhouse, M.E.J. (2004). Acquisition and epidemiology of antibiotic resistant *Escherichia coli* in a cohort of new-born calves. *Journal of Antimicrobial Chemotherapy* **53**, 867-871.

Hughes, J.B., Hellmann, J.J., Rickets, T.H., and Bohannan, B.J. (2001). Counting the uncountable: Statistical approaches to estimating microbial diversity. *Applied and Environmental Microbiology* **67**, 4399-4406.

Huletsky, A., Knox, J.R., and Levesque, R.C. (1993). Role of Ser-238 and Lys-240 in the hydrolysis of third-generation cephalosporins by SHV-type beta-lactamases probed by site-directed mutagenesis and three-dimensional modelling. *Journal of Biological Chemistry* **268**, 3690-3697.

Hunter, J.E.B., Shelley, J.C., Walton, J.R., Hart, C.A., and Bennett, M. (1992). Apramycin resistance plasmids in *Escherichia coli*: possible transfer to *Salmonella typhimurium* in calves. *Epidemiology and Infection* **108**, 271-278.

Hunter, J.E.B., Hart, C.A., Shelley, J.C., Walton, J.R., and Bennett, M. (1993). Human isolates of apramycin-resistant *Escherichia coli* which contain the genes for the AAC(3)IV enzyme. *Epidemiology and Infection* **110**, 253-259.

Hunter, J.E.B., Bennett, M., Hart, C.A., Shelley, J.C., and Walton, J.R. (1994). Apramycin-resistant *Escherichia coli* isolated from pigs and a stockman. *Epidemiology and Infection* **112**, 473-480.

Hutchison, J.G.P. (1954). Antibiotic sensitivity tests: A rapid method suitable for multiple cultures. *Journal of Clinical Pathology* **7**, 350-351.

Ichige, A.S., Matsutani, S., Oishi, K., and Mizushima, S. (1989). Establishment of gene transfer systems and construction of the genetic map of a marine *Vibro* strain. *Journal of Bacteriology* **171**, 1825-1834.

Inselburg, J. (1978). ColE1 plasmid mutants affecting growth of an *Escherichia coli recB recC sbcC* mutant. *Journal of Bacteriology* **133**, 433-436.

Jeong, S.H., Bae, I.K., Kwon, S.B., Lee, J.H., Jung, H.I., Song, J.S., Jeong, B.C., Kim, S.J., and Lee, S.H. (2004a). Investigation of extended-spectrum beta-lactamases produced by clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Korea. *Letters in Applied Microbiology* **39**, 41-47.

Jeong, S.H., Bae, I.K., Lee, J.H., Sohn, S.G., Kang, G.H., Jeon, G.J., Kim, Y.H., Jeong, B.C., and Lee, S.H. (2004b). Molecular characterization of extended-spectrum beta-lactamases produced by clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* from a Korean nationwide survey. *Journal of Clinical Microbiology* **42**, 2902-2906.

Jiang, S.C., and Paul, J.H. (1998). Gene transfer by transduction in the marine environment. *Applied and Environmental Microbiology* **64**, 2780-2787.

Johnson, A.P., Burns, L., Woodford, N., Threlfall, E.J., Naidoo, J., Cooke, E.M., and George, R.C. (1994). Gentamicin resistance in clinical isolates of *Escherichia coli* encoded by genes of veterinary origin. *Journal of Medical Microbiology* **40**, 221-226.

Johnson, A.P., Malde, M., Woodford, N., Cunney, R.J., and Smith, E.G. (1995). Urinary isolates of apramycin-resistant *Escherichia coli* and *Klebsiella pneumoniae* from Dublin. *Epidemiology and Infection* **114**, 105-112.

Johnson, J.R., and O'Bryan, T.T. (2000). Improved repetitive-element PCR fingerprinting for resolving pathogenic and non-pathogenic phylogenetic groups within *Escherichia coli*. *Clinical and Diagnostic Laboratory Immunology* **7**, 265-273.

Johnston, M. (1998). Use of antimicrobial drugs in veterinary practice. *British Medical Journal* **317**, 665-667.

Jones, I.M., Primrose, S.B., Robinson, A., and Ellwood, D.C. (1980). Maintenance of some ColE1-type plasmids in chemostat culture. *Molecular and General Genetics* **180**, 579-584.

Jones, R.N. (2000). Detection of emerging resistance patterns within longitudinal surveillance systems: data sensitivity and microbial susceptibility. *Journal of Antimicrobial Chemotherapy* **46**, Suppliment B, 1-8.

Juni, E., and Janik, A. (1969). Transformation of *Acinetobacter calco-aceticus* (*Bacterium anitratum*). *Journal of Bacteriology* **98**, 281-288.

Katouli, M., Lund, A., Wallgren, P., Kuhn, I., Soderland, O., and Mollerby, R. (1995). Phenotypic characterisation of intestinal *Escherichia coli* of pigs during suckling, postweaning, and fattening periods. *Applied and Environmental Microbiology* **61**, 778-783.

Kayama, A.M., and Yara, T. (1975). Plasmid mutations affecting self-maintenance and host growth in *Escherichia coli*. *Journal of Bacteriology* **122**, 80-88.

Kerrn, M.B., Klemmensen, T., Frimodt-Moller, N., and Espersen, F. (2002). Susceptibility of Danish *Escherichia coli* strains isolated from urinary tract infections and bacteraemia, and distribution of *sul* genes conferring sulphonamide resistance. *Journal of Antimicrobial Chemotherapy* **50**, 513-516.

Khachatourians, G.G. (1998). Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. *Canadian Medical Association Journal* **159**, 1129-1136.

Klimke, W.A., and Frost, L.S. (1998). Genetic analysis of the role of the transfer gene, traN, of the F and R100-1 plasmids in mating pair stabilization during conjugation. *Journal of Bacteriology* **180**, 4036-4043.

Kluytmans, J., Vanleeuwen, W., Goessens, W., Hollis, R., Messer, S., Herwaldt, L., Bruining, H., Heck, M., Rost, J., van Leeuwen, N., van Belkum, A., and Verbrugh, H. (1995). Food-initiated outbreak of methicillin-resistant *Staphylococcus aureus* analyzed by phenotyping and genotyping. *Journal of Clinical Microbiology* **33**, 1121-1128.

Knox, J.R. (1995). Extended-spectrum and inhibitor-resistant TEM-type β-lactamases: Mutations, specificity, and three-dimensional structure. *Antimicrobial Agents and Chemotherapy* **39**, 2593-2601.

Koeth, L.M., King, A., Knight, H., May, J., Miller, L.A., Phillips, I., and Poupard, J.A. (2000). Comparison of cation-adjusted Mueller-Hinton broth with Iso-Sensitiest broth for the NCCLS broth microdilution method. *Journal of Antimicrobial Chemotherapy* **46**, 369-376.

Kruse, H., and Sørum, H. (1994). Transfer of multiple-drug resistance plasmids between bacteria of diverse origins in natural microenvironments. *Applied and Environmental Microbiology* **60**, 4015-4021.

Krause, D.O., Smith, W.J.M., Conlan, L.L., Gough, J.M., Williamson, A., and McSweeney, C.S. (2003). Diet influences the ecology of lactic acid bacteria and *Escherichia coli* along the digestive tract of cattle: neural networks and 16SrDNA. *Microbiology* **149**, 57-65.

Lacks, S., Greenberg, B., and Neuberger, M. (1975). Identification of a deoxyribonuclease implicated in genetic transformation of *Diplococcus pneumoniae*. *Journal of Bacteriology* **123**, 222-232.

Landis, R.J., and Koch, G.G. (1977). The measurement of observer agreement for categorical data. *Biometrics* **33**, 159-174.

Le Bouguenec, C., de Cespedes, G., and Horaud, T. (1990). Presence of chromosomal elements resembling the composite structure TN3701 in *Streptococci*. *Journal of Bacteriology* **172**, 727-734.

Lee, S.W., and Edlin, G. (1985). Expression of tetracycline resistance in pBR322 derivatives reduces the reproductive fitness of plasmid-containing *Escherichia coli*. *Gene* **39**, 173-180.

Lee, K., Yong, D., Yum, J.H., Kim, H.H., and Chong, Y. (2003). Diversity of TEM-52 extended-spectrum ß-lactamase-producing non-typhoidal *Salmonella* isolates in Korea. *Journal of Antimicrobial Chemotherapy* **52**, 493-496.

Leflon-Guibout, V., Speldooren, V., Heym, B., and Nicolas-Chanoine, M. (2000). Epidemiological survey of amoxicillin-clavulanate resistance and corresponding molecular mechanisms in *Escherichia coli* isolates in France: new genetic features of bla(TEM) genes. *Antimicrobial Agents and Chemotherapy* **44**, 2709-2714.

Lenski, R.E., Rose, M.R., Simpson, S.C., and Tadler, S.C. (1991). Long-term experimental evolution in *Escherichia coli*. 1. Adaption and divergence during 2,000 generations. *The American Naturalist* **138**, 1315-1341.

Lenski, R.E., Simpson, A.C., and Nguyen, T.T. (1994). Genetic analysis of a plasmid-encoded, host genotype-specific enhancement of bacterial fitness. *Journal of Bacteriology* **176**, 3140-3147.

Levin, B.R., Stewart, F.M., and Rice, V.A. (1979). The kinetics of conjugative plasmid transmission: fit of a simple mass action model. *Plasmid* **2**, 247-260.

Levin, B.R., Perrot, V., and Walker, N. (2000). Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. *Genetics* **154**, 985-997.

Levy, S.B., and McMurry. L. (1974). Detection of an inducible membrane protein associated with R-factor-mediated tetracycline resistance. *Biochemical and Biophysical Research Communications* **56**, 1060-1068.

Levy, S.B. (1976). Spread of antibiotic-resistance plasmids from chicken to chicken and from chicken to man. *Nature* **260**, 40-42.

Levy, S.B. (1987). Antibiotic use and antibiotic resistance worldwide. *Reviews of Infectious Diseases* **9** (supplement 1), 231-316.

Levy, S.B. (1998) Antimicrobial resistance: bacteria on the defence. *British Medical Journal* **317**, 612-613.

Licht, T.R., Christensen, B.B., Krogfelt, K.A., and Molin, S. (1999). Plasmid transfer in the animal intestine and other dynamic bacterial populations: the role of community structure and environment. *Microbiology* **145**, 2615-2622.

Linton, A.H. (1977a). Antibiotic resistance: the present situation reviewed. *Veterinary Record* **100**, 354-360.

Linton, A.H. (1977b). Animal to man transmission of Enterobacteriaceae. *Royal Society of Health Journal* **97**, 115-118.

Linton, A.H. (1984). Antibiotic-resistant bacteria in animal husbandry. *British Medical Bulletin* **40**, 91-95.

Lipsitch, M., and Levin, B.R. (1997). The population dynamics of antimicrobial chemotherapy. *Antimicrobial Agents and Chemotherapy* **41**, 363-373.

Livermore, D.M., and Chen, H.Y. (1999). Quality of antimicrobial susceptibility testing in the UK: a *Pseudomonas aeruginosa* survey revisited. *Journal of Antimicrobial Chemotherapy* **43**, 517-522.

Livermore, D.M., Stephens, P., and Weinberg, J., Johnson, A.P., Gifford, T., Northcott, D., James, D., George, R.C., and Speller, D.C. (2000). Regional variation in ampicillin and trimethoprim resistance in *E. coli* in England from 1990 to 1997, in relation to antibacterial prescribing. *Journal of Antimicrobial Chemotherapy* **46**, 411-422.

Livermore, D.M., and Brown, D.F. (2001). Detection of beta-lactamase-mediated resistance. *Journal of Antimicrobial Chemotherapy* **48**, Supplement 1, 59-64.

Maison, T.G., and Richardson, G. (1981). A review: *Escherichia coli* and the human gut: Some ecological considerations. *Journal of Applied Bacteriology* **1**, 1-16.

Marshall, B., Petrowski, D., and Levy, S.B. (1990). Inter- and intraspecies spread of *Escherichia coli* in a farm environment in the absence of antibiotic usage. *Proceedings of the National Academy of Sciences* USA. **87**, 6609-6613.

Martin, I.E., Tyler, S.D., Tyler, K.D., Khakharia, R., and Johnson, W.M. (1996). Evaluation of ribotyping as epidemiological tool for typing *Escherichia coli* serogroup O157 isolates. *Journal of Clinical Microbiology* **34**, 720-723.

Martin, A.P. (2002). Phylogenetic approaches for describing and comparing the diversity of microbial communities. *Applied and Environmental Microbiology* **68**, 3673-3682.

Martinez, J.L., Alonso, A., Gomez-Gomez, J.M., and Baquero, F. (1998). Quinolone resistance by mutations in chromosomal gyrase genes. Just the tip of the iceberg? *Journal of Antimicrobial Chemotherapy* **42**, 683-688.

Masterton, R.G. (2000). Surveillance studies: how can they help the management of infection? *Journal of Antimicrobial Chemotherapy* **46**, Supplement T2, 53-58.

Mathis, L.S., and Scocca, J.J. (1982). *Haemophilus influenzae* and *Neisseria gonorrhoeae* recognize different specificity determinants in the DNA uptake step of genetic transformation. *Journal of General Microbiology* **128**, 1159-1161.

Mazodier, P., and Davies , J. (1991). Gene transfer between distantly related bacteria. *Annual Review of Genetics* **25**, 147-171.

McCaig, A.E., Glover, L.A., and Prosser, J.I. (1999). Molecular analysis of bacterial community structure and diversity in improved and unimproved upland grass pastures. *Applied and Environmental Microbiology* **65**, 1721-1730.

McCracken, A., O'Brien, J. J., and Campbell, N. (1976). Antibiotic residues and their recovery from animal tissues. *Journal of Applied Bacteriology* **41**, 129-135.

McGeer, A.J. (1998). Agricultural antibiotics and resistance in human pathogens: Villain or scapegoat? *Canadian Medical Association Journal* **159**, 1119-1120.

Mee, B.J., and Nikoletti, S.M. (1983). Plasmids encoding trimethoprim resistance in bacterial isolates from man and pigs. *Journal of Applied Bacteriology* **54**, 225-235.

Modi, R.I., Castilla, L.H., Puskas-Rozsa, S., Helling, R.B., and Adams, J. (1992). Genetic changes accompanying increased fitness in evolving populations of *Escherichia coli. Genetics* **130**, 241-249.

Monod, J. (1949). The growth of bacterial cultures. *Annual Review of Microbiology* **2**, 371-394.

Moosden, F. (1997). The evolution of resistance to cephalosporins. *Clinical Infectious Diseases* **24**, 487-493.

Muela, A., Pocino, M., Arana, I., Justo, J.I., Iriberri, J., and Barcina, I. (1994). Effect of growth phase and parental cell survival in river water on plasmid transfer between *Escherichia coli* strains. *Applied and Environmental Microbiology* **60**, 4273-4278.

Mueller, U.G., and Wolfenbarger, L.L. (1999). AFLP genotyping and fingerprinting. *Trends in Ecology and Evolution* **14**, 389-394.

Mugnier, P., Podglajen, I., Goldstein, F.W., and Collatz, E. (1998). Carbapenems as inhibitors of OXA-13, a novel, integron-encoded beta-lactamase in *Pseudomonas aeruginosa. Microbiology* **144**, 1021-1031.

Murray, N.E. (2002). Immigration control of DNA in bacteria: self versus non-self. *Microbiology* **148**, 3-20.

Naas, T., Zerbib, M., Girlich, D., and Nordmann, P. (2003). Integration of a transposon Tn1-encoded inhibitor-resistant beta-lactamase gene, bla(TEM-67) from *Proteus mirabilis*, into the *Escherichia coli* chromosome. *Antimicrobial Agents and Chemotherapy* **47**, 19-26.

Negri, M., Lipstitch, M., Blazquez, J., Levin, B.R., and Baquero, F. (2000). Concentration-dependant selection of small phenotypic differences in TEM β-lactamase-mediated antibiotic resistance. *Antimicrobial Agents and Chemotherapy* **44**, 2485-2491.

Nelson, E.C., and Elisha, B.G. (1999). Molecular basis of AmpC hyperproduction in clinical isolates of *Escherichia coli. Antimicrobial Agents and Chemotherapy* **43**, 957-959.

Nijsten, R., London, N. and Van den Bogaard, A., and Stobberingh, E. (1995). *In-vivo* transfer of resistance plasmids in rat, human or pig-derived intestinal flora using a rat model. *Journal of Antimicrobial Chemotherapy* **36**, 975-985.

Nijsten, R., London, N., Van den Bogaard, A., and Stobberingh, E. (1996). Antibiotic resistance among *Escherichia coli* isolated from faecal samples of pig farmers and pigs. *Journal of Antimicrobial Chemotherapy* **37**, 1131-1140.

Nikaido, H., Basina, M., Nguyen, V., Rosenberg, E.Y. (1998). Multidrug efflux pump AcrAB of *Salmonella typhimurium* excretes only those β-lactam antibiotics containing lipophilic side chains. *Journal of Bacteriology* **180**, 4686-4692.

Nikolich, M.P., Shoemaker, N.B., Wang, G.R., and Salyers, A.A. (1994a). Characterisation of a new type of *Bacteroides* conjugative transposon, TC-R-EM(R)-7853. *Journal of Bacteriology* **176**, 6606-6612.

Nikolich, M.P., Hong, G., Shoemaker, N.B., and Salyers, A.A. (1994b). Evidence for natural horizontal transfer of TetQ between bacteria that normally colonise humans and bacteria that normally colonise livestock. *Applied and Environmental Microbiology* **60**, 3255-3260.

Noack, D., Roth, M., Guether, R., Muller, G., Undisz, K., Hoffmeier, C., and Gaspar, S. (1981). Maintenance and genetic stability of vector plasmids pBR322 and pBR325 in *Escherichia coli* K12 strains grown in a chemostat. *Molecular and General Genetics* **184**, 121-124.

Noller, A.C., McEllistrem, M.C., Stine, O.C., Morris, J.G.Jr., Boxrud, D.J., Dixon, B., and Harrison, L.H. (2003). Multilocus sequence typing reveals a lack of diversity among *Escherichia coli* O157:H7 isolates that are distinct by pulsed-field gel electrophoresis. *Journal of Clinical Microbiology* **41**, 675-679.

O'Brien, T.F. (1997). The global epidemic nature of antimicrobial resistance and the need to monitor and manage it locally. *Clinical Infectious Diseases* **24**, Supplement 1, S2-8.

Ochman, H., and Selander, R.K. (1984). Evidence for clonal population structure in *Escherichia coli. Proceedings of the National Academy of Sciences, USA* **81**, 198-201.

Oethinger, M., Jellen-Ritter, A.S., Conrad, S., Marre, R., and Kern, W.V. (1998). Colonisation and infection with fluoroquinolone-resistant *Escherichia coli* among cancer patients: Clonal analysis. *Infection* **26**, 379-384.

Ofek, I., Mirelman, D., and Sharon, N. (1977). Adherence of *Escherichia coli* to human mucosal cells mediated by mannose receptors. *Nature* **265**, 623-625.

Okusu, H., Ma, D., and Nikaido, H. (1996). AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. *Journal of Bacteriology* **178**, 306-308.

Olsen, R,H., Siak, J. and Gray, R.H (1974). Characteristics of PrD1, a plasmid dependant broad host range DNA bacteriophage. *Journal of Virology* **14**, 689-699.

Oppegaard, H., Steinum, T.M., and Wasteson, Y. (2001). Horizontal transfer of a multi-drug resistance plasmid between coliform bacteria of human and bovine origin in a farm environment. *Applied and Environmental Microbiology* **67**, 3732-3734.

Oram, M., and Fisher, L.M. (1991). 4-Quinolone resistance mutations in the DNA gyrase of *Escherichia coli* clinical isolates identified by using the polymerase chain reaction. *Antimicrobial Agents and Chemotherapy* **35**, 387-389.

Orman, B.E., Pineiro, S.A., Arduino, S., Galas, M., Melano, R., Caffer, M.I., Sordelli, D.O., and Centron, D. (2002). Evolution of multiresistance in nontyphoid *salmonella* serovars from 1984 to 1998 in Argentina. *Antimicrobial Agents and Chemotherapy* **46**, 3963-3970.

Ørskov, F., Whittam, T.S., Cravioto, A., and Orskov, I. (1990). Clonal relationships among classic enteropathogenic *Escherichia coli* (EPEC) belonging to different O groups. *The Journal of Infectious Diseases* **162**, 76-81.

Osek, J. (2000). Clonal analysis of *Escherichia coli* strains isolated from pigs with post-weaning diarrhea by pulsed-field gel electrophoresis. *FEMS Microbiology Letters* **186**, 327-331.

Österblad, M., Hakanen, A. Manninen, R., Leistevuo, T., Peltonen, R., Meurman, O., Huovinen, P., and Kotilainen, P. (2000). A between-species comparison of antimicrobial resistance in enterobacteria in fecal flora. *Antimicrobial Agents and Chemotherapy* **44**, 1479-1484.

Papaparaskevas, J., Vatopoulos, A., Tassios, P.T., Avlami, A., Legakis, N.J., and Kalapothaki, V. (2000). Diversity among high-level aminoglycoside-resistant enterococci. *Journal of Antimicrobial Chemotherapy* **45**, 277-283.

Parsonnet, K.C., and Kass, E.H. (1987). Does prolonged exposure to antibiotic-resistant bacteria increase the rate of antibiotic-resistant infection? *Antimicrobial Agents and Chemotherapy* **31**, 911-914.

Pearce, B.J., Naughton, A.M., Campbell, E.A., and Masure, H.R. (1995). The rec locus, a competence-induced operon in *Streptococcus pneumoniae*. *Journal of Bacteriology* **177**, 86-93.

Perilli, M., Dell'Amico, E., Segatore, B., Rosaria de Massis, M., Bianchi, C., Luzzaro, F., Rossolini, G.M., Toniolo, A., Nicoletti, G., and Amicosante, G. (2002). Molecular characterization of extended-spectrum β-lactamases produced by nosocomial isolates of Enterobacteriacae from an Italian nationwide survey. *Journal of Clinical Microbiology* **40**, 611-614.

Perlada, D.E., Smulian, A.G., Cushion, M.T. (1997). Molecular epidemiology and antibiotic susceptibility of enterococci in Cincinnati, Ohio: a prospective citywide survey. *Journal of Clinical Microbiology* **35**, 2342-2347.

Pezzella, C., Ricci, A., DiGiannatale, E., Luzzi, I., and Carattoli, A. (2004). Tetracycline and streptomycin resistance genes, transposons, and plasmids in *Salmonella enterica* isolates from animals in Italy. *Antimicrobial Agents and Chemotherapy* **48**, 903-908.

Philippon, A., Arlet, G., and Jacoby, G.A. (2002). Plasmid-determined AmpC-type β-lactamases. *Antimicrobial Agents and Chemotherapy* **46**, 1-11.

Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C., Preson, R., and Waddell, J. (2004). Does the use of antibiotics in food pose a risk to human health? A critical review of published data. *Journal of Antimicrobial Chemotherapy* **53**, 28-52.

Piddock, L.J.V. (1996). Does the use of antimicrobial agents in veterinary medicine and animal husbandry select antibiotic-resistant bacteria that infect man and compromise antimicrobial chemotherapy? *Journal of Antimicrobial Agents and Chemotherapy* **38**, 1-3.

Pitout, J.D., Hanson, N.D., Church, D.L., and Laupland, K.B. (2004). Population-based laboratory surveillance for *Escherichia coli*-producing extended-spectrum beta-lactamases: importance of community isolates with blaCTX-M genes. *Clinical Infectious Diseases* **38**, 1736-1741.

Platt, D.J., Smith, I. (1991). Gentamicin-resistant *Salmonella typhimurium* phage type 204C: molecular studies and strain diversity in a putative bovine outbreak. *Epidemiology and Infection* **107**, 213-217.

Pohl, P., Glupczynski, Y., Marin M., Van Robaeys, G., Lintermans, P., Couturier, M. (1993). Replicon typing characterization of plasmids encoding resistance to gentamicin and apramycin in *Escherichia coli* and *Salmonella typhimurium* isolated from human and animal sources in Belgium. *Epidemiology and Infection* **111**, 229-238.

Poulsen, L.K., Lan, F., Kristensen, C.S., Hobolth, P., Molin, S., and Krogfelt, K.A. (1994). Spatial distribution of *Escherichia coli* in the mouse large intestine inferred from rRNA *in situ* hybridisation. *Infection and Immunity* **62**, 5191-5194.

Pournaras, S., Ikonomidis, A., Kristo, I., Tsakris, A., and Maniatis, A.N. (2004). CTX-M enzymes are the most common extended-spectrum beta-lactamases among *Escherichia coli* in a tertiary Greek hospital. *Journal of Antimicrobial Chemotherapy* **54**, 574-575.

Power, E.G.M. (1996). RAPD typing in microbiology – a technical review. *Journal of Hospital Infection* **34**, 247-265.

Poyart, C., Mugnier, P., Quesne, G., Berche, P., and Trieu-Cuot, P. (1998). A novel extended-spectrum TEM-type beta-lactamase (TEM-52) associated with decreased susceptibility to moxalactam in *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy* **42**, 108-113.

Provence, D.L., and Curtiss, R. (1994). Gene transfer in gram-negative bacteria. In: Gerhardt P, murray RGE, Wood WA, Kreig N (eds*) Methods for general and molecular biology*. American Society for Microbiology, Washington, DC, pages 338-339.

Quale, J., Bratu, S., Landman, D., Heddurshetti, R. (2003). Molecular epidemiology and mechanisms of carbapenem resistance in *Acinetobacter baumannii* endemic in New York City. *Clinical Infectious Diseases* **37**, 214-220.

Rådstrom, P., Swedberg, G., and Skold, O. (1991). Genetic analyses of sulfonamide resistance and its dissemination in Gram-negative bacteria illustrate new aspects of R plasmid evolution. *Antimicrobial Agents and Chemotherapy* **35**, 1840-1848.

Randall, L.P., Cooles, S.W., Sayers, A.R., Woodward, M.J. (2001). Association between cyclohexane resistance in *Salmonella* of different serovars and increased resistance to multiple antibiotics, disinfectants and dyes. *Journal of Medical Microbiology* **50**, 910-924.

Recchia, G.D., and Hall, R.M. (1995). Gene cassettes: A new class of mobile element. *Microbiology* **141**, 3015-3027.

Reynolds, M.G. (2000). Compensatory evolution in rifampin-resistant *Escherichia coli. Genetics* **156**, 1471-1481.

Rios, M., Prado, V., Trucksis, M., Arellano, C., Borie, C., Alexandre, M., Fica, A., and Levine, M.M. (1999). Clonal diversity of Chillean isolates of enterohemorrhagic *Escherichia coli* from patients with haemolytic-uremic syndrome, asymptomatic subjects, animal reservoirs and food products. *Journal of Clinical Microbiology* **37**, 778-781.

Rivera, M.J., Rivera, N., Castillo, J., Rubio, M.C., and Gomez-Lus, R. (1991). Molecular and epidemiological study of Salmonella clinical isolates. *Journal of Clinical Microbiology* **29**, 927-932.

Roberts, M.C. (1990). Characterisation of the TetM determinants in urogenital and respiratory bacteria. *Antimicrobial Agents and Chemotherapy* **34**, 476-478.

Roberts, M.C., Pang, Y.J., Spencer, R.C., Winstanley, T.G., Brown, B.A., and Wallace, R.J.Jr. (1991). Tetracycline resistance in *Moraxella-(branhamella)-catarrhalis* - demonstration of two clonal outbreaks by using pulsed-field gel-electrophoresis. *Antimicrobial Agents and Chemotherapy* **35**, 2453-2455.

Rosenberg, E., Ma, D., and Nikaido, H. (2000). AcrD of *Escherichia coli* is an aminoglycoside efflux pump. *Journal of Bacteriology* **182**, 1754-1756.

Roy, P.H. (1999). Horizontal transfer of genes in bacteria. *Microbiology today* **26**, 168-170.

Rudin, L., Sjostrom, J.E., Lindberg, M., and Philipson, L. (1974). Factors affecting competence for transformation in *Staphylococcus aureus. Journal of Bacteriology* **118**, 155-164.

Salauze, D., Otal, I., Gomez-lus, R., and Davies, J. (1990). Aminoglycoside acetyltransferase 3-IV (*aaC4*) and hygromycin B 4-I phosphotransferase (*hphB*) in bacteria isolated from human and animal sources. *Antimicrobial Agents and Chemotherapy* **34**, 1915-1920.

Salisbury, J.G., Nicholls, T.J., Lammerding, A.M., Turnidge, J., and Nunn, M.J. (2002). A risk analysis framework for the long-term management of antibiotic resistance in food-producing animals. *International Journal of Antimicrobial Agents* **20**, 153-164.

Salyers, A.A., and Shoemaker, N.B (1996). Resistance gene transfer in anaerobes: New insights, new problems. *Clinical Infectious Diseases* **23**, S36-S43.

Salyers, A.A., and Amabile-Cuevas, S.F. (1997). Why are antibiotic resistance genes so resistant to elimination? *Antimicrobial Agents and Chemotherapy* **41**, 2321-2325.

Salyers, A.A (1999a). Beware the commensals! Their role in antibiotic resistance spread may be greater than we think. *ASM News* **65**, 459-469.

Salyers, A.A. (1999b). Agricultural use of antibiotics and antibiotic resistance in human pathogens: Is there a link? Reservoirs of Antibiotic resistance (ROAR) report. www.healthsci.tufts.edu/apua/roarhome.htm

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proeedings of the National Academy of Sciences, USA* **74**, 5463-5467.

Schiappa, D.A., Hayden, M.K., Matushek, M.G., Hashemi, F.N., Sullivan, J., Smith, K.Y., Miyashiro, D., Quinn, J.P., Weinstein, R.A., and Trenholme, G.M. (1996). Ceftazidime-resistant *Klebiella pneumoniae* and *Escherichia coli* bloodstream infection: a case-control and molecular epidemiologic investigation. *Journal of Infectious Diseases* **174**, 529-536.

Scholz, P., Haring, V., Wittmann-Liebold, B., Ashman, K., Bagdasarian, M., and Scherzinger, E. (1989). Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. *Gene* **75**, 271-288.

Schrag, S.J, Perrot, V., and Levin, B.R. (1997). Adaption to the fitness costs of antibiotic resistance in *Escherichia coli. Proceedings of the Royal Society of London Series B-Biological Sciences* **264**, 1287-1291.

Schwarz, S., Kehrenberg, C., and Walsh, T.R. (2001). Use of antimicrobial agents in veterinary medicine and food animal production. *International Journal of Antimicrobial Agents* **17**, 431-437.

Scott, J.R. (1992). Sex and the single circle - conjugative transposition. *Journal of Bacteriology* **174**, 6005-6010.

Sears, H.J., and Brownlee, I. (1952). Further observations on the persistence of individual strains of *Escherichia coli* in the intestinal tract of man. *Journal of Bacteriology* **63**, 47-57.

Selander, R.K., and Levin, B.R. (1980). Genetic diversity and structure in *Escherichia coli* populations. *Science* **210**, 545-547.

Selander, R.K., Caugant, D.A., Ochman, H., Musser, J.M., Gilmour, M.N. and Whittam, T.S. (1986). Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Applied and Environmental Microbiology* **51**, 873-884.

Select Committee on Science and Technology (1998). House of Lords Seventh Report. www.parliment.the-stationary-office.htm

Seurinck, S., Verstraete, W., and Siciliano, S.D. (2003). Use of 16S-23S rRNA intergenic spacer region PCR and repetitive extragenic palindromic PCR analyses of *Escherichia coli* isolates to identify nonpoint fecal sources. *Applied and Environmental Microbiology* **69**, 4942-4950.

Shanahan, P.M., Thomson, C.J., and Amyes, S.G.B. (1995) Beta-lactam resistance in normal faecal flora from South Africa. *Epidemiology and Infection* **115**, 243-253.

Shaw K.J., Rather P.N., Hare R.S., and Millwe, G.H. (1993). Molecular genetics of aminoglycoside resistance genes and familial reslationships of the aminoglycoside-modifying enzymes. *Microbiological Reviews* **57**, 138-163.

Shedlofsky, S., and Freter, R. (1974). Synergism between ecological and immunological control mechanisms of intestinal flora. *Journal of Infectious Disease* **129**, 296-303.

Shen, D., Biedenbach, D.J., Winokur, P.L., Pfaller, M.A. and Jones, R.N. (1999). Phenotypic and genotypic characterizations of Chinese strains of *Escherichia coli* producing extended-spectrum β-lactamases. *Diagnostic Microbiology and Infectious Diseases* **34**, 159-164.

Shoemaker, N.B., Smith, M.D., and Guild, W.R. (1980). DNAse-resistant transfer of chromosomal *cat* and *tet* insertions by filter mating in pneumococcus. *Plasmid* **3**, 80-87.

Shoemaker, N.B., Getty, C., Guthrie, E.P., and Salyers, A.A. (1986). Regions in *Bacteroides* plasmids pBFTM10 and pB8-51 that allow *Escherichia coli-Bacteroides* shuttle vectors to be mobilized by IncP plasmids and by a conjugative *Bacteroides* tetracycline resistance element. *Journal of Bacteriology* **166**, 959-965.

Sigler, W.V., and Zeyer, J. (2002). Microbial diversity and activity along the forefold of two receeding glaciers. *Microbial Ecology* **43**, 397-407.

Silveira, W.D., Lancellotti, M., Ferreira, A., Solferini, V.N., Castro, A.F.P., Stehling, E.G. and Brocchi, M. (2003). Determination of the clonal structure of avian *Escherichia coli* strains by isoenzyme and ribotyping analysis. *Journal of Veterinary Medicine* **50**, 63-69.

Simonsen, L., Gordon D.M., Steward, F.M., and Levin, B.R. (1990). Estimating the rate of plasmid transfer: an end-point method. *Journal of General Microbiology* **136**, 2319-2325.

Simpson, I.N., Harper, P.B., and O'Callaghan, C.H. (1980). Principal beta-lactamases responsible for resistance to beta-lactam antibiotics in urinary tract infections. *Antimicrobial Agents and Chemotherapy* **17**, 929-936.

Skirrow, M.B. (1977). *Campylobacter enteritidis*: a "new" disease. *British Medical Journal* **ii**: 9-11.

Smets, B.F., Rittermann, B.E., and Stahl, D.A. (1993). The specific growth rate of *Pseudomonas putida* PAW1 influences the conjugal transfer rate of the TOL plasmid. *Applied and Environmental Microbiology* **59**, 3430-3437.

Smith, H.E., and Crabb, W.E. (1961). The faecal bacterial flora of animals and man: its development in the young. *Journal of Pathology and Bacteriology* **82**, 53-66.

Smith, H.W. (1975). Persistence of tetracycline resistance in pig *E. coli*. *Nature* **258**, 628-630.

Smith, H.O., Danner, D.B., and Deich, R.A. (1981). Genetic transformation. *Annual Review of Biochemistry* **50**, 41-68.

Sokal, R.R., and Rohlf, F.J. (1995). *Biometry*. 3rd Ed. W.H. Freeman and Company, New York.

Son, R., Rusul, G., and Karim, M.I.A. (1997). Conjugal transfer of plasmids and antibiotic resistance among *Escherichia coli* isolated from animals in a rural area in Sarawak (Malaysia). *Journal of Applied Microbiology* **82**, 240-244.

Spika, J.S., Blake, P.A., and Cohen, M.L. (1987). Chloramphenicol-resistant *Salmonella-newport* traced through hamburger to dairy farms. *New England Journal of Medicine* **317**, 632-632.

Stanier, R.Y., Adelberg, E.A., and Ingraham, J.L. (1983). Symbiotic associations between two nonphotosynthetic partners. In: *General Microbiology*. 4th Edition. The Macmillan Press Ltd. London and Basingstoke (UK). Chapter 28, p782-783.

Steward, C.D., Stocker, S.A., Swenson, J.M., O'Hara, C.M., Edwards, J.R., Gaynes, R.P., McGowan, J.E. Jr., and Tenover, F.C. (1999). Comparison of agar dilution, disc diffusion, MicroScan, and Vitek antimicrobial susceptibility testing methods to broth microdilution for detection of fluoroquinolone-resistant isolates of the familiy *Enterobacteriaceae. Journal of Clinical Microbiology* **37**, 544-547.

Sulavik, M.C., Houseweart, C., Cramer C., Jiwani, N., Murgolo, N., Greene, J., Di Domenico, B., Shaw, K.J., Miller, G.H., Hare, R., and Shimer, G. (2001). Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes. *Antimicrobial Agents and Chemotherapy* **45**, 1126-1136.

Swann Committee (1969). Report of the joint committee on the use of antibiotics in animal husbandry and veterinary medicine, HMSO. http://www.parliament.the-stationery-office.co.uk/pa/ld199798/ldselect/ldsctech/081vii/st0701.htm Date last accessed: August 9th 2004.

Swartley, J.S., Mcallister, C.F., Hajjeh, R.A., Heinrich, D.W., and Stephens, D.S. (1993). Deletions of TN916-like transposons are implicated in tetM-mediated resistance in pathogenic *Neisseria. Molecular Microbiology* **10**, 299-310.

Tarkka, E., Ahman, H., and Siitonen, A. (1994). Ribotyping as an epidemiologic tool for *Escherichia coli Epidemiology and Infection* **112**, 263-274.

Tascini, C., Menichetti, F., Bozza, S., Fedele, M., Preziosi, R., Allegrucci, M., Del Favero, A., Micozzi, A., Martino, P., and Bistoni, F. (1999). Molecular typing of fluoroquinolone-resistant and fluoroquinolone-susceptible *Escherichia coli* isolated from blood of neutropenic cancer patients in a single centre. *Clinical Microbiology and Infection* **5**, 457-461.

Tenney, J.H., Maack, R.W., and Chipendale, G.R. (1983). Rapid selection of organisms with increasing resistance on subinhibitory concentrations of norfloxacin in agar. *Antimicrobial Agents and Chemotherapy* **23**, 188-189.

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelson, P.A., Murray, B.E., Persing, D.H., and Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: Criteria for bacterial strain typing. *Journal of Clinical Microbiology* **33**, 2233-2239.

Tenover, F.C., Mohammed, M.J., Stelling, J., O'Brien, T., and Williams, R. (2001). Ability of laboratories to detect emerging antimicrobial resistance: Proficiency testing and quality control results from the World Health Organization's external quality assurance system for antimicrobial susceptibility testing. *Journal of Clinical Microbiology* **39**, 241-250.

Teuber, M., and Perreten, V. (2000). Role of milk and meat products as vehicles for antibiotic- resistant bacteria. *Acta Veterinaria Scandinavica* **93**,75-87.

Thomas, D.J.I., Morgan, A.W., Whipps, J.M., and Saunders, J.R. (2001). Plasmid transfer between *Bacillus thuringiensis* subsp. *israelensis* strains in laboratory culture, river water, and dipteran larvae. *Applied and Environmental Microbiology* **67**, 330-338.

Thomson, C.J., Tait, S., Jesudason, M.V., Amyes, S.G.B. (1993). Dissemination of the TEM-I beta-lactamase gene into disparate plasmids in faecal strains of *Escherichia coli* isolated in Vellore, south India. *Journal of Infection* **27**, 47-50.

Threlfall, E. J., Frost, J. A., Ward, L. R., and Rowe, B. (1994). Epidemic in cattle and humans of *Salmonella-typhimurium* DT-104 with chromosomally integrated multiple-drug resistance. *Veterinary Record* **134**, 577-577.

Threlfall, E.J. (2002). Antimicrobial drug resistance in *Salmonella*: problems and perspectives in food- and water-borne infections. *FEMS Microbiology Reviews* **26**, 141-148.

Timoney, J.F., and Linton, A.H. (1982). Experimental ecological-studies on H-2 plasmids in the intestine and feces of the calf. *Journal of Applied Bacteriology* **52**, 417-424.

Toivanen, P., Vaahtovuo, J., and Eerola, E. (2001). Influence of major histocompatibility complex on bacterial composition of faecal flora. *Infection and Immunity* **69**, 2372-2377.

Towner, K.J., Venning, B.M., and Pinn, P.A. (1982). Occurrence of transposable trimethoprim resistance in clinical isolates of *Escherichia coli* devoid of self-transmissible resistance plasmids. *Antimicrobial Agents and Chemotherapy* **21**, 336-338.

Traub, P., and Nomura, M. (1986). Streptomycin resistance mutation in *Escherichia coli*: altered ribosomal protein. *Science* **160**, 198-199.

Tzouvelekis, L.S., Tzelepi, E., Tassios, P.T., and Legakis, N.J. (2000). CTX-M-type beta-lactamases: an emerging group of extended-spectrum enzymes. *International Journal of Antimicrobial Agents* **14**, 137-42.

Vahaboglu, H., Fuzi, M., Cetin, S., Gundes, S., Ujhelyi, E., Coskunkan, F., and Tansel, O. (2001). Characterization of extended-spectrum ß-lactamase (TEM-52) producing strains of *Salmonella enterica* Serovar Typhimurium with diverse resistance phenotypes. *Journal of Clinical Microbiology* **39**, 791-793.

Valentine, P.J., Shoemaker, N.B., and Salyers, A.A (1988). Mobilization of *Bacteroides* plasmids by *Bacteroides* conjugal elements. *Journal of Bacteriology* **170**, 1319-1324.

Vali, L., Wisely, K.A., Pearce, M.C., Turner, E.J., Knight, H.I., Smith, A.W., and Amyes, S.G.B. (2004). High genotypic variations and antibiotic sensitivity among *Escherichia coli* O157 strains isolated from two Scottish beef cattle farms. *Applied and Environmental Microbiology*. In Press.

Van Belkum, A., Bax, R., Peerbooms, P., Goessens, W.H., van Leeuwen, N., and Quint, W.G. (1993). Comparison of phage typing and DNA fingerprinting by polymerase chain reaction for discrimination of methicillin-resistant *Staphylococcus aureus* strains. *Journal of Clinical Microbiology* **31**, 798-803.

Van den Bogaard, A.E., Jenson, L.B., and Stobberingh, E.E. (1997). Vancomycin-resistant enterococci in turkeys and farmers. *New England Journal of Medicine* **337**, 1558-1559.

Van den Bogaard, A.E. and Stobberingh, E.E. (2000). Epidemiology of resistance to antibiotics - Links between animals and humans. *International Journal of Antimicrobial Agents* **14**, 327-335.

Van den Bogaard, A.E., Bruinsma N., and Stobberingh, E.E (2000). The effect of banning avoparcin on VRE carriage in The Netherlands. *Journal of Antimicrobial Chemotherapy* 46, 146-148.

Verma, V., Qazi, G.N., Parshad, R., and Chopra, C.L. (1989). Introduction of a *Micrococcus* plasmid in *Escherichia coli. Plasmid* **22**, 265-267.

Versalovic, J., Koeuth, T., and Lupski, J.R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes *Nucleic Acids Research* **19**, 6823-6831.

Veterinary Medicines Directorate (2002). Sales of antimicrobial products authorised for use as veterinary medicines, antiprotozoals, antifungals, growth promoters and coccidiostats and in the UK in 2002. Report of the Veterinary Medicines Directorate 2002. http://www.vmd.gov.uk/general/publications/salesanti02.pdf Date last accessed, 9th August 2004.

Vila, J., Marcos, A., Marco, F., Abdalla, S., Vergara, Y., Reig, R., Gomez-Lus, R., and Jimenez de Anta, T. (1993). In vitro antimicrobial production of beta-lactamases, aminoglycoside-modifying enzymes, and chloramphenicol acetyltransferase by and susceptibility of clinical isolates of *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy* **37**, 138-141.

Vila J., Ruiz J., Marco F., Barcelo, A., Goni, P., Giralt, E., and Jimenez de Anta, T. (1994). Association between double mutation in gyrA gene of ciprofloxacin-resistant clinical isolates of *Escherichia coli* and MICs. *Antimicrobial Agents and Chemotherapy* **38**, 2477-2479.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**, 4407-4414.

Walmsley, R.H. (1976). Temperature dependence of mating-pair formation of *Escherichia coli*. *Journal of Bacteriology* **126**, 222-224.

Wang, Y., and Taylor, D.E. (1990). Natural transformation in *Campylobacter* species. *Journal of Bacteriology* **172**, 949-955.

Wang, M., Tran, J.H., Jacoby, G.A., Zhang, Y., Wang, F., and Hooper, D.C. (2003). Plasmid-mediated quinolone resistance in clinical isolates of *Escherichia coli* from Shanghai, China. *Antimicrobial Agents and Chemotherapy* **47**, 2242-2248.

Welsh, J., and McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* **18**, 7213-7218.

Wheat, P.F. (2001). History and development of antimicrobial susceptibility testing methodology. *Journal of Antimicrobial Chemotherapy* **48**, *Supplement S1*, 1-4.

Whittam, T.S., Wolfe, M.L., Wachsmuth, I.K., Orskov, F., Orskov, I., and Wilson, R.A. (1993). Clonal relationships among *Escherichia coli* that cause hemorrhagic colitis and infantile diarrhea. *Infection and Immunity* **61**, 1619-1629.

Wiener, J., Quinn, J.P., Bradford, P.A., Goering, R.V., Nathan, C., Bush, K., and Weinstein, R.A. (1999). Multiple antibiotic-resistant *Klebsiella* and *Escherichia coli* in nursing homes. *Journal of the American Medical Association* **281**, 517-523.

Wierup, M. (2001). The Swedish experience of the 1986 year ban of antimicrobial growth promoters, with special reference to animal health, disease prevention, productivity, and use of antimicrobials. *Microbial Drug Resistance* **7**, 183-190.

Winokur, P.L., Canton, R., Cassellas, J.M., Legakis, N. (2001). Variations in the prevalence of strains expressing an extended-spectrum beta-lactamase phenotype and characterization of isolates from Europe, the Americas, and the Western Pacific region. *Clinical Infectious Diseases* **15**, *Supplement 2*, S94-103.

Wise, E.M.Jr. and Abou-Donia, M.M. (1975). Sulfonamide resistance mechanism in *Escherichia coli*: R plasmids can determine sulfonamide-resistant dihydropteroate synthases. *Proceedings of the National Academy of Science, USA*. **72**, 2621-5.

World Health Organisation (1997). *The medical impact of the use of antimicrobials in food animals*, Report of a WHO meeting, Berlin, Germany.

Wray, C., Hedges, R.W., Shannon, K.P., and Bradley, D.E. (1986). Apramycin and gentamicin resistance in *Escherichia coli* and salmonellas isolated from farm animals. *Journal of Hygiene: Cambridge* **97**, 445-456.

Yagi, T., Kruokawa, H., Shibata, N., Shibayama, K., and Arakawa, Y. (2000). A preliminary survey of extended-spectrum β-lactamases (ESBLs) in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Japan. *FEMS Microbiology Letters* **184**, 53-56.

Yamamoto, T., Yamagata, S., Horii, K., and Yamagishi, S. (1982). Comparison of transcription of beta-lactamase genes specified by various ampicillin transposons. *Journal of Bacteriology* **150**, 269-276.

Yang, H.H., Vinopal, R.T., Grasso, D., and Smetts, B.F. (2004). High diversity among environmental *Escherichia coli* isolates from a bovine feedlot. *Applied and Environmental Microbiology* **70**, 1528-1536.

Young, H.K., and Amyes, S.G.B. (1986). A new mechanism of plasmid trimethoprim resistance. Characterization of an inducible dihydrofolate reductase. *The Journal of Biological Chemistry* **261**, 2503-2505.

Zelazna-Kowalska, I. (1977). [^{3}H] dihydrostreptomycin accumulation and binding to ribosomes in *Rhizobium* mutants with different levels of streptomycin resistance. *Journal of Bacteriology* **132**, 8-12.

Zeph, L.R., Onaga, M.A., and Stotzky, G. (1988). Transduction of *Escherichia coli* by bacteriophage P1 in soli. *Applied and Environmental Microbiology* **54**, 1731-1737.

Zhao, S., White, D.G., McDermott, P.F., Friedman, S., English, L., Ayers, S., Meng, J., Maurer, J.J., Holland, R., and Walker, R.D. (2001). Identification and expression of cepamycinase bla_{CMY} genes in *Escherichia coli* and *Salmonella* isolates from food animals and ground meat. *Antimicrobial Agents and Chemotherapy* **45**, 3647-3650.

Zund, P., and Lebek, G. (1980). Generation time-prolonging R plasmids: correlation between increases in the generation time of *Escherichia coli* caused by R plasmids and their molecular size. *Plasmid* **3**, 65-69.

Appendix 1. Results of a sensitivity analysis to determine how different parameters influence the output of the plasmid transfer in batch culture model.

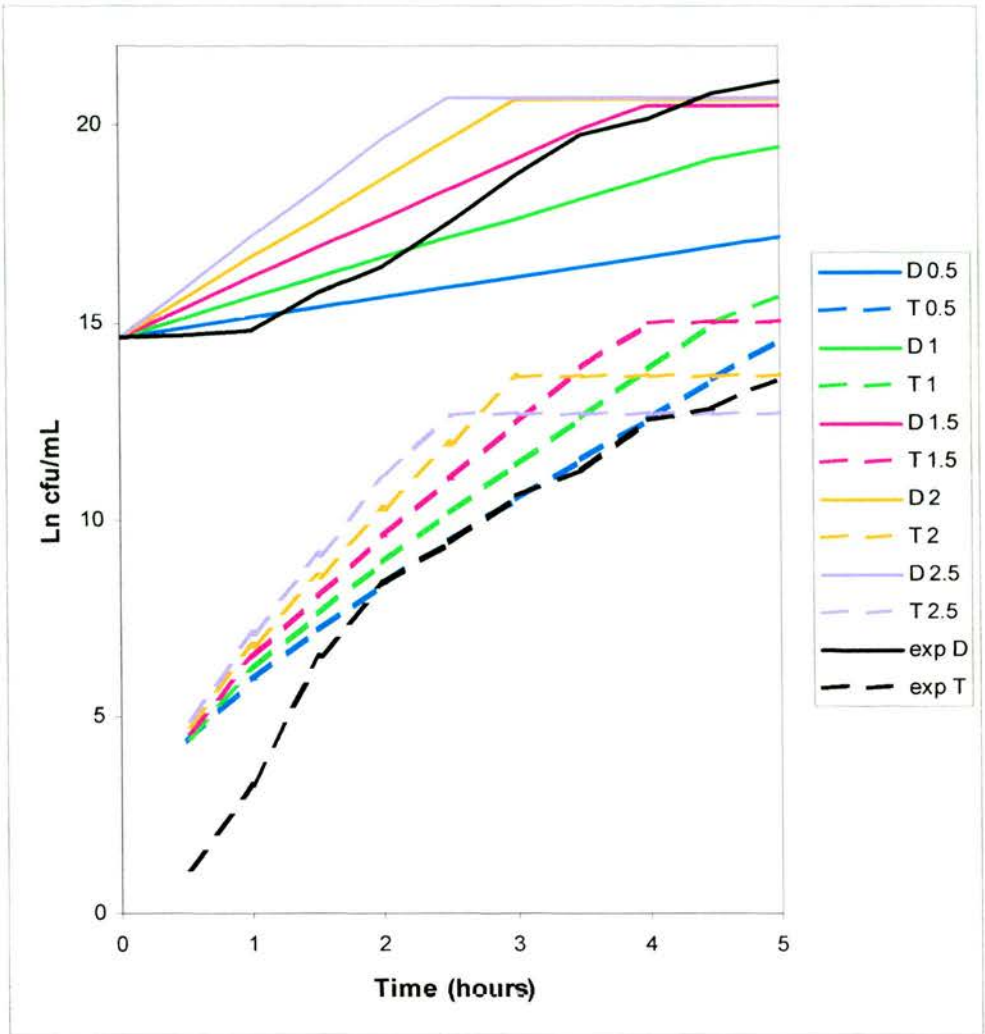


Figure A1. The effect of donor growth rate on the transconjugant population during the transfer of pUK2002 between J53 and J62-2.

Figure A1 demonstrates that the best fit for the T population is a low donor growth rate (vD) (vD of 0.5 cfu/mL), but this results in very inaccurate modelling of the D population. Additionally, resource limitation is reached at too low a cell density compared to the experimental data, indicating that the model should be run with a greater amount of resource than that currently used.

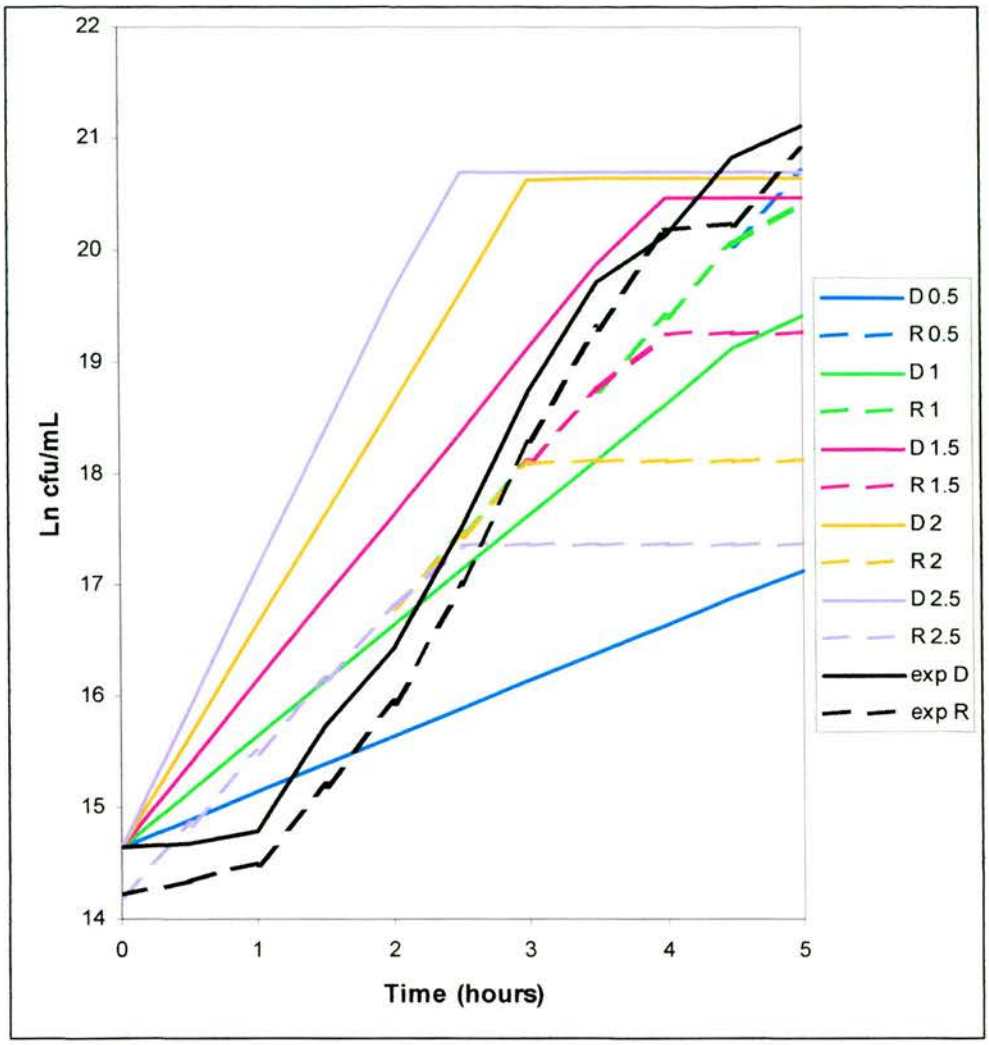


Figure A2. The effect of donor growth rate on the recipient population during the transfer of pUK2002 between J53 and J62-2.

The effect of donor growth rate (vD) on R (Figure A2) is one of direct competition. When vD > vR, D constitutes a greater proportion of the total population at resource limitation than R, and vice versa.

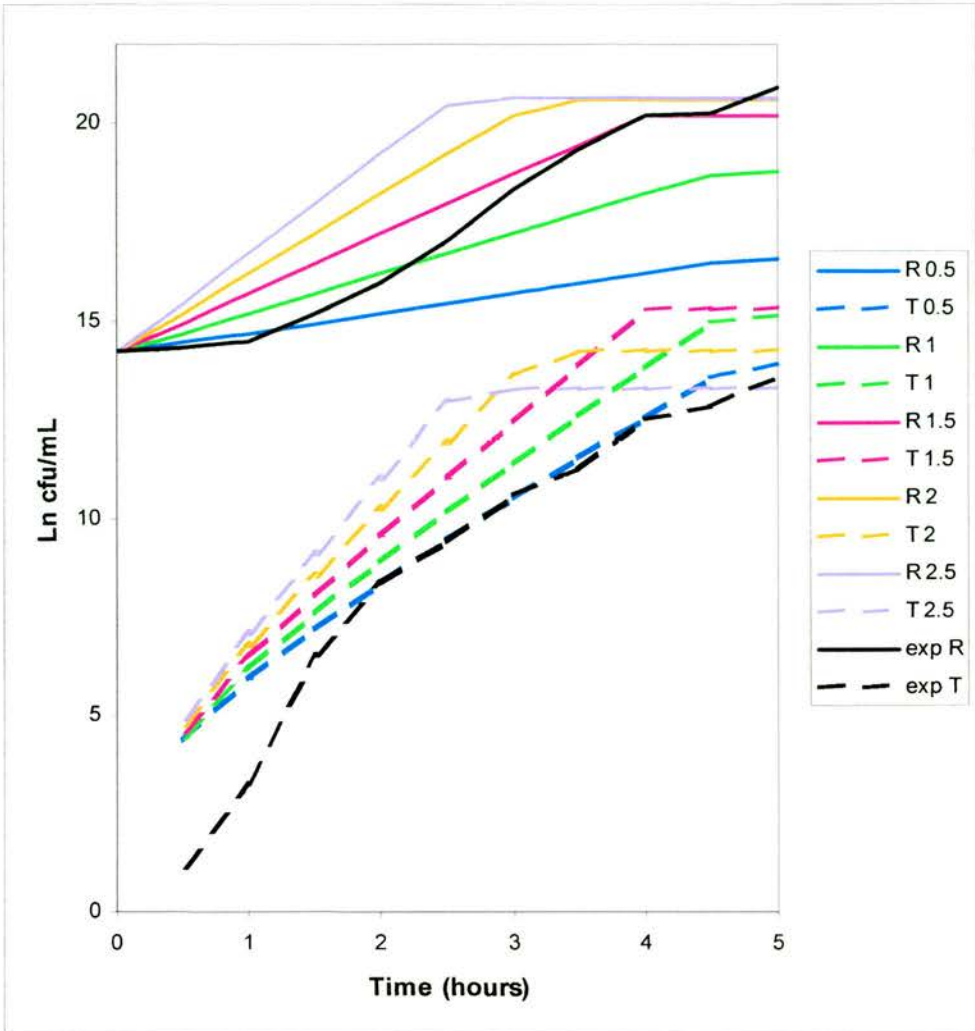


Figure A3. The effect of recipient growth rate on the transconjugant population during the transfer of pUK2002 between J53 and J62-2.

Figure A3 is almost identical to that of the effect of vD on T (Figure A1), with the best fit of the experimental T population being achieved with the lowest recipient growth rate (vR), 0.5. The use of a low vR results in inaccurate modelling of R.

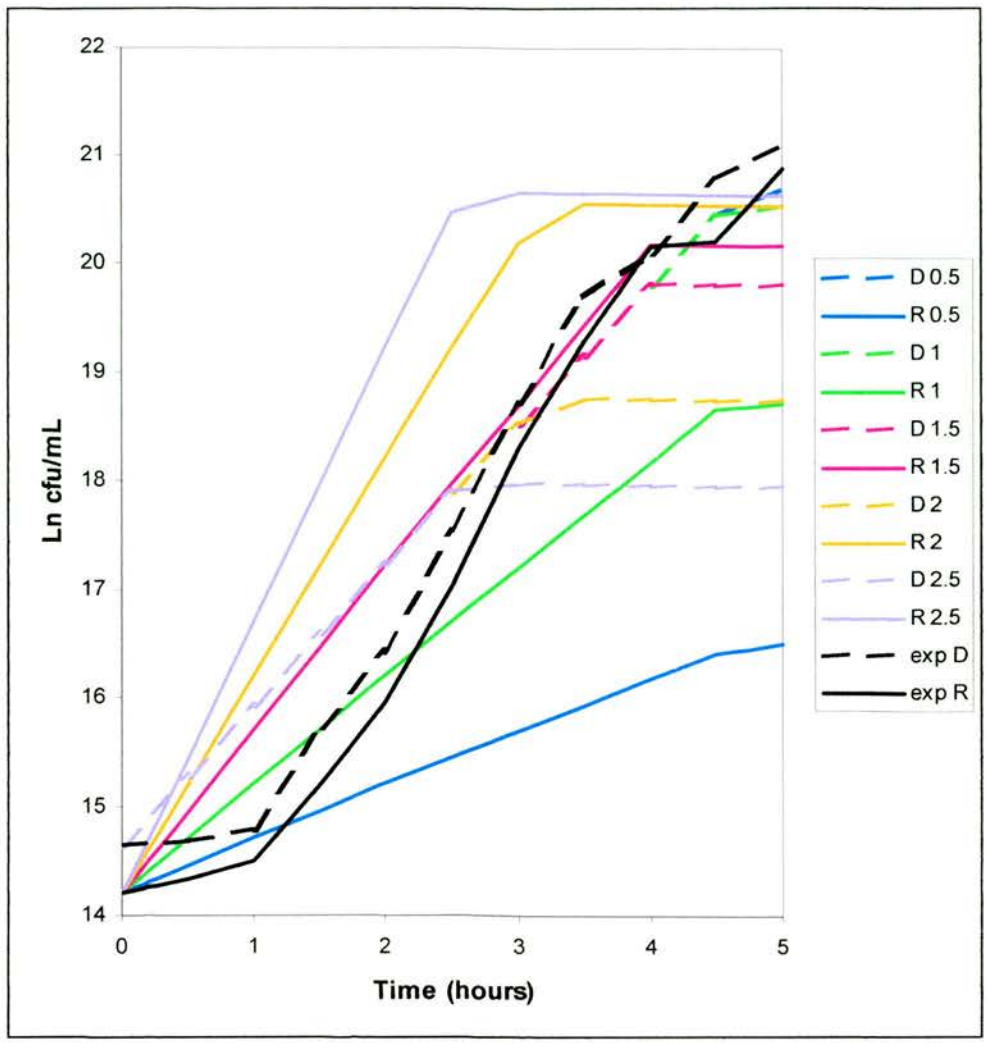


Figure A4. The effect of recipient growth rate on the donor population during the transfer of pUK2002 between J53 and J62-2.

In a similar way to the effect of vD on R (Figure A1), if vR > vD, R will occupy a greater proportion of the broth than D (Figure A4). The reduction in D as a function of increasing vR is less than the reduction in R due to increasing vD because D only acts in competition with the other strains in the broth, whereas R is affected by conversion to T at a rate determined by γ, in addition to the effect of competition with D and T.

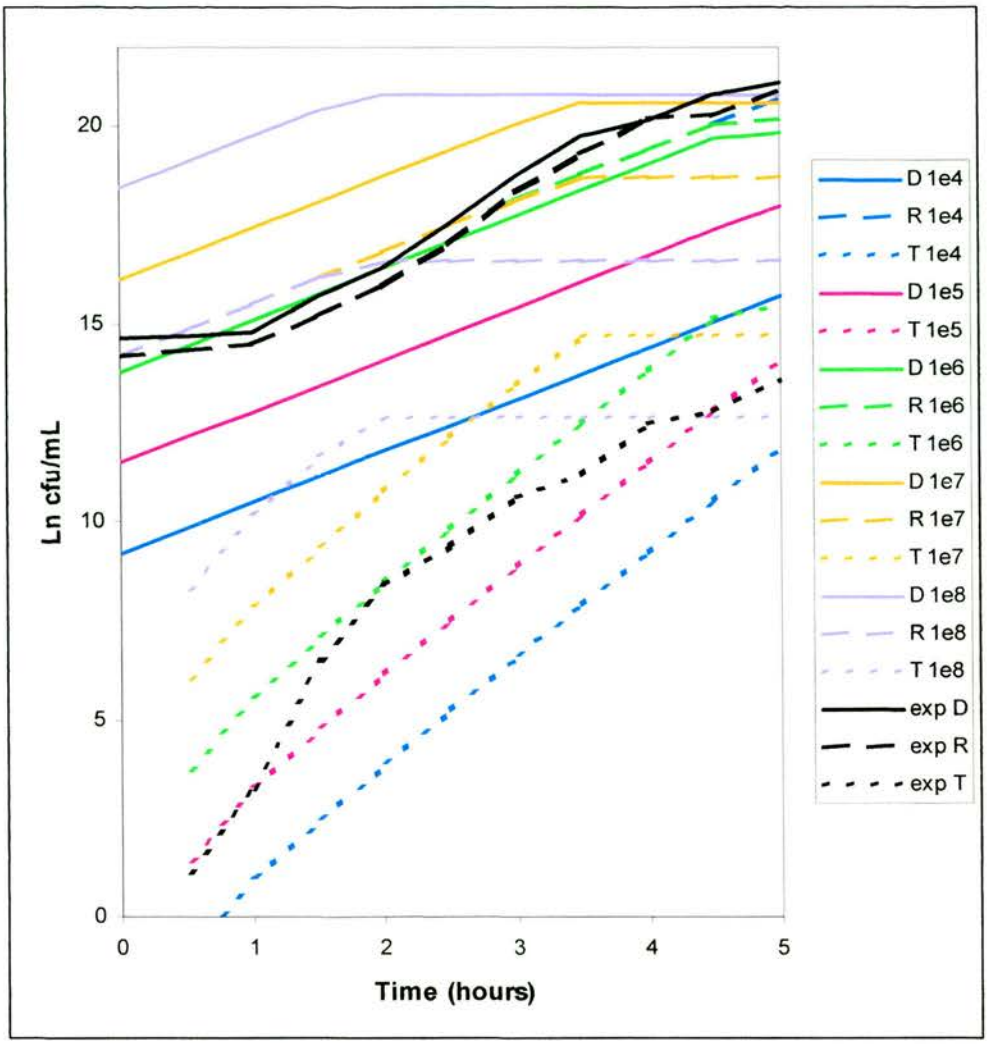


Figure A5. The effect of different initial D densities on the population dynamics of pUK2002 transfer.

When the initial density of D (iD) is greater than initial density of R (iR) the density of R is not affected throughout the experiment (Figure A5). When iD > iR, D reaches resource limitation before R, therefore R occupied a smaller proportion of the broth than D throughout the experiment. When the model was run with different initial densities of R, similar results were obtained in that when iR < iD, the density of D is not affected but when iR > iD, R occupies a greater proportion of broth than D (results not shown). Lowering iD or iR produces the same T population curve, but at lower cell densities.

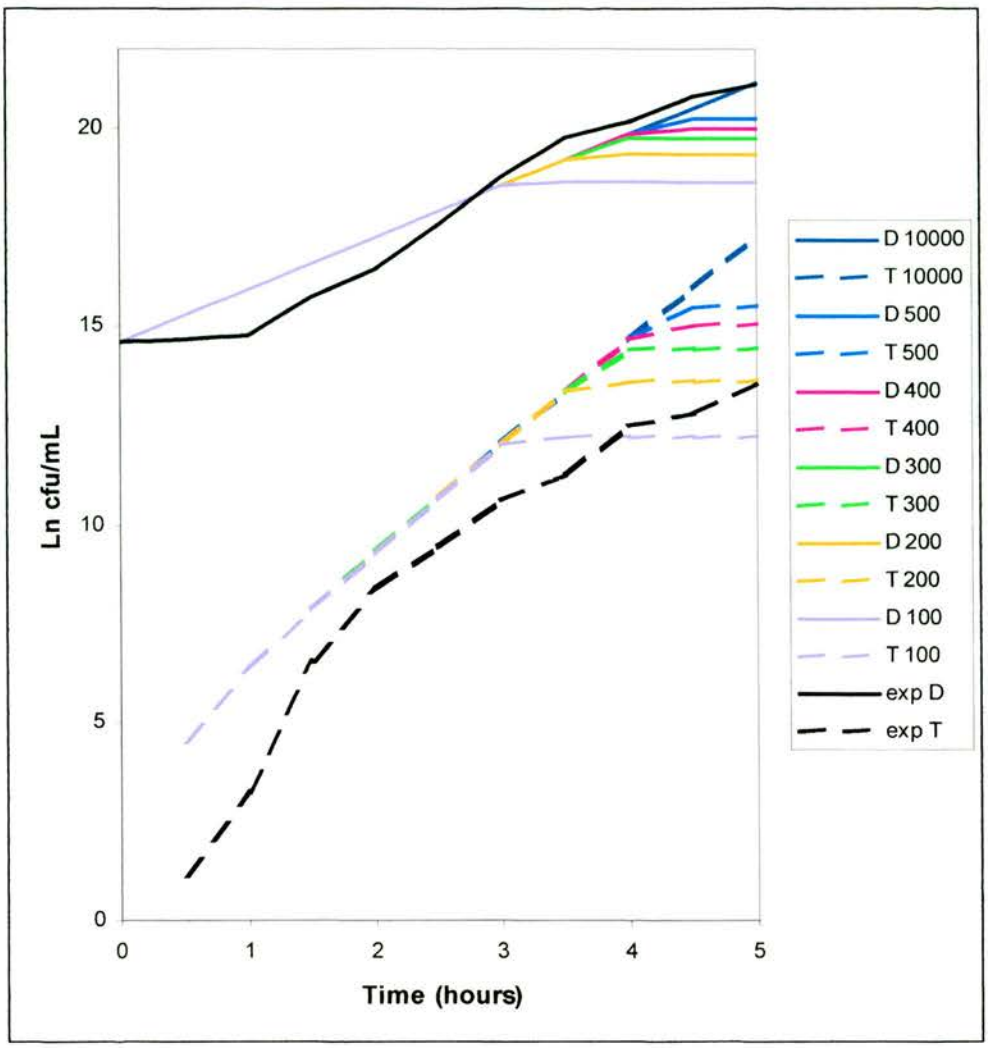


Figure A6. The effect of resource limitation on the population dynamics of pUK2002 plasmid transfer. The resource concentrations used are listed in the key as D or T and the concentration of glucose added (200-1000).

There is a trade off between an increase in resource concentration, enhancing the fit of the donor (and recipient) populations, but resulting in a reduced fit to the transconjugant population. This graph also makes it clear that the model is unable to take into account an initial lag in growth of the donors (and recipients). The sigmoid curve of these populations is not reproduced by the model, which assumes bacterial growth is constant and exponential from the moment of initial inoculation.

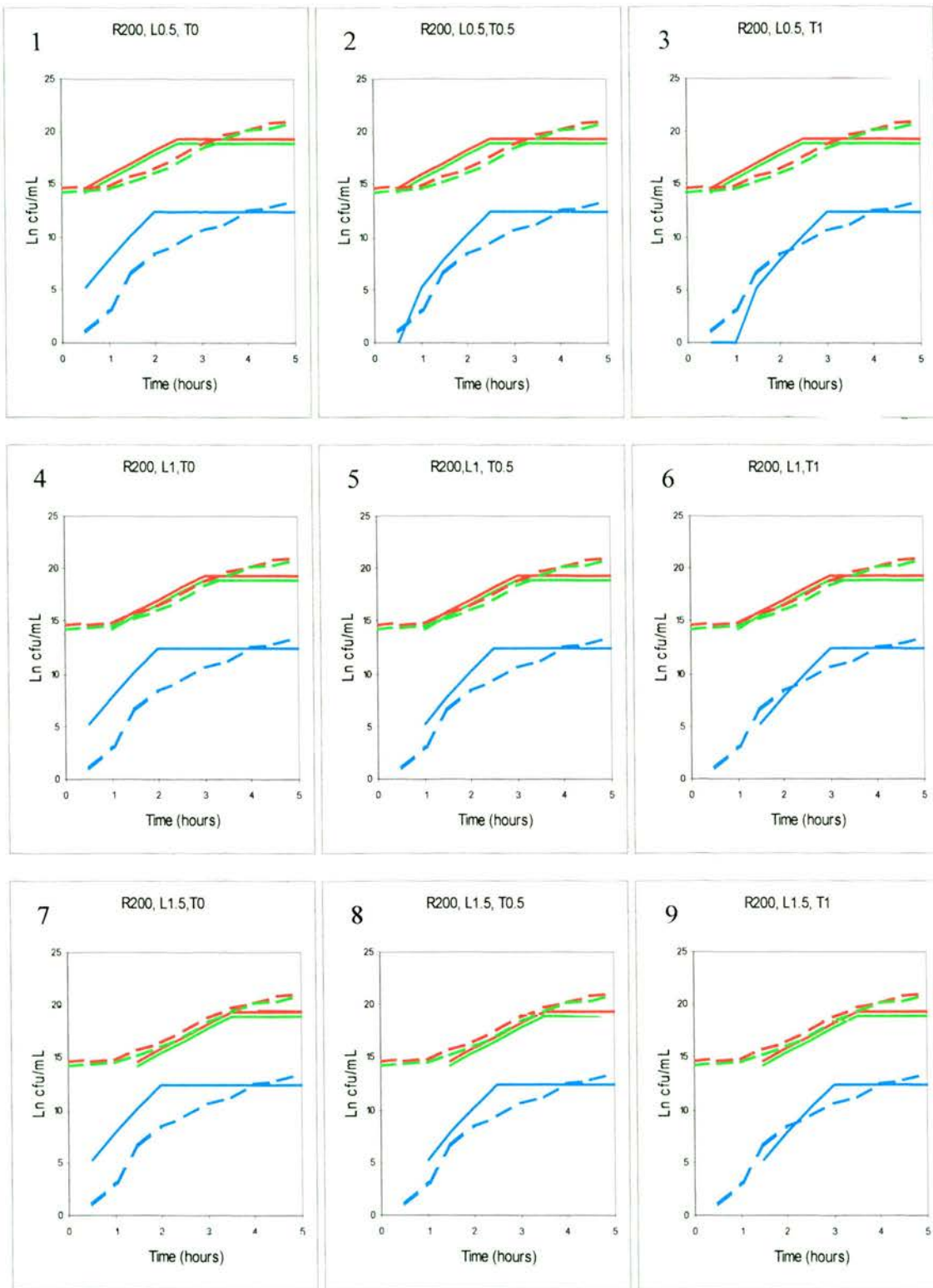
Appendix 2. Results of different combinations of the three parameters (resource concentration, lag in D and R growth, and lag in transfer) found to enhance the fit of the model to the experimental data.

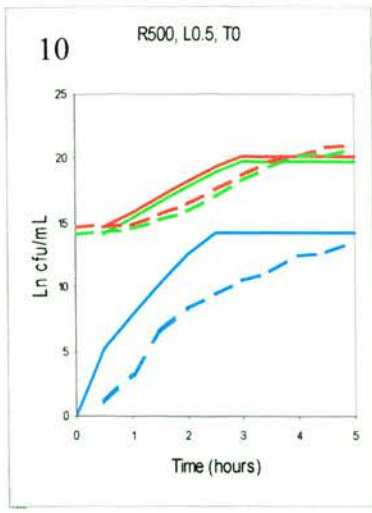
Three different values of each of the three parameters were tested. In each of the following graphs (1-27) the value of each parameter is listed in the title according to the following key:

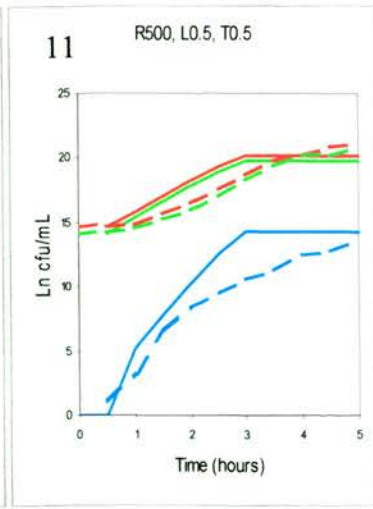
R200; 200μg/mL of resource.
R500; 500μg/mL of resource.
R1000; 1000μg/mL of resource.

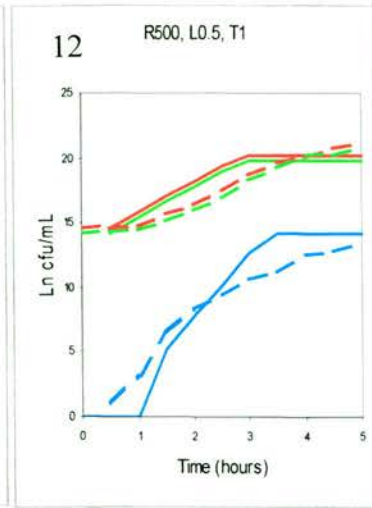
L0.5; 05h lag before D and R growth.
L1; 1h lag before D and R growth.
L1.5; 1.5h lag before D and R growth.

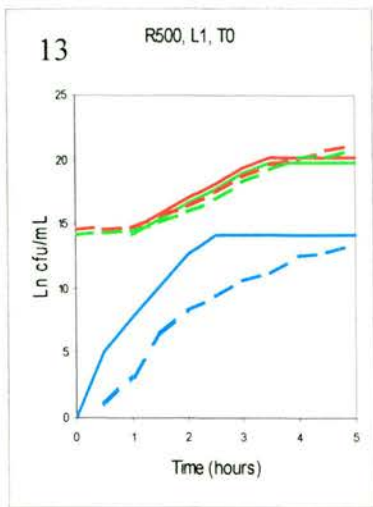
T0; 0h lag before plasmid transfer.
T0.5; 0.5h lag before plasmid transfer.
T1; 1h lag before plasmid transfer.

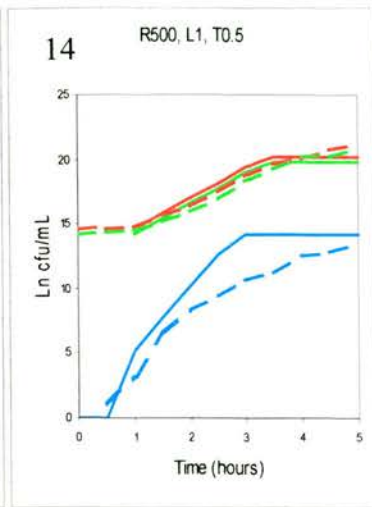

1
R200, L0.5, T0
2
R200, L0.5,T0.5
3
R200, L0.5, T1
4
R200, L1,T0
5
R200,L1, T0.5
6
R200, L1,T1
7
R200, L1.5,T0
8
R200, L1.5, T0.5
9
R200, L1.5, T1
Ln cfu/mL
Time (hours)

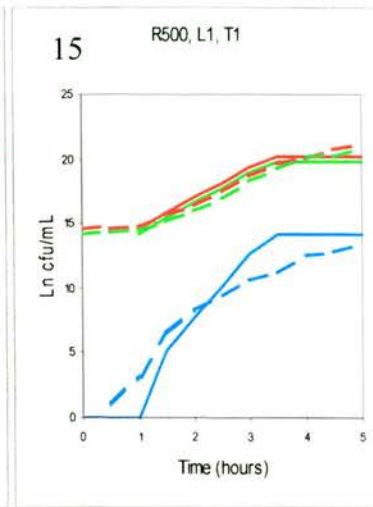

10
R500, L0.5, T0
Ln cfu/mL
Time (hours)

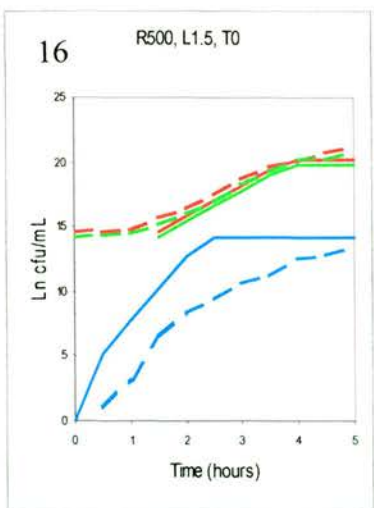

11
R500, L0.5, T0.5
Ln cfu/mL
Time (hours)

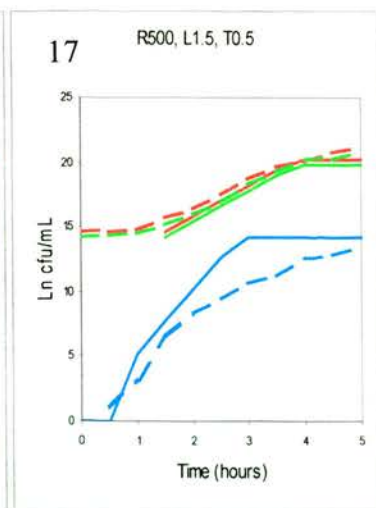

12
R500, L0.5, T1
Ln cfu/mL
Time (hours)

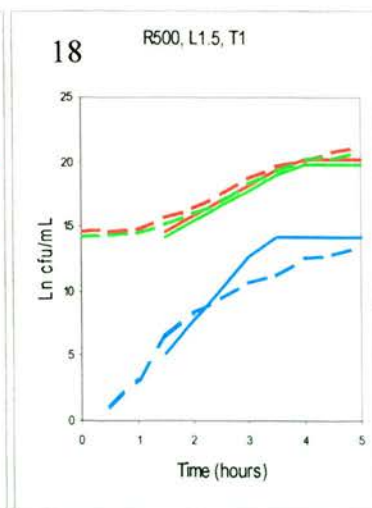

13
R500, L1, T0
Ln cfu/mL
Time (hours)


14
R500, L1, T0.5
Ln cfu/mL
Time (hours)

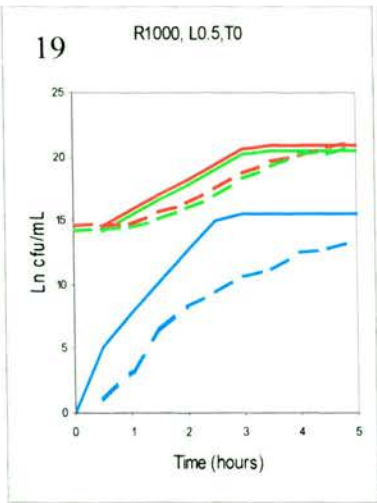

15
R500, L1, T1
Ln cfu/mL
Time (hours)


16
R500, L1.5, T0
Ln cfu/mL
Time (hours)

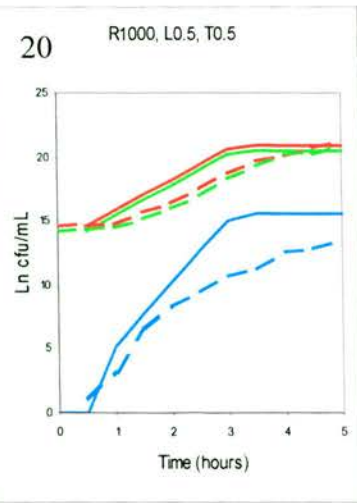

17
R500, L1.5, T0.5
Ln cfu/mL
Time (hours)


18
R500, L1.5, T1
Ln cfu/mL
Time (hours)

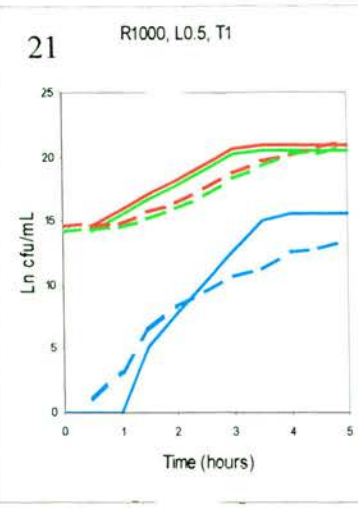
19



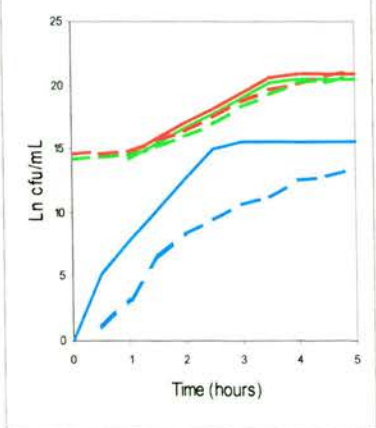
20



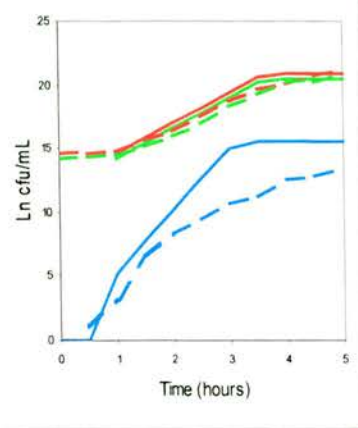
21



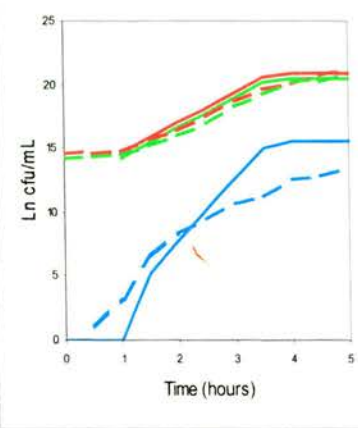
22



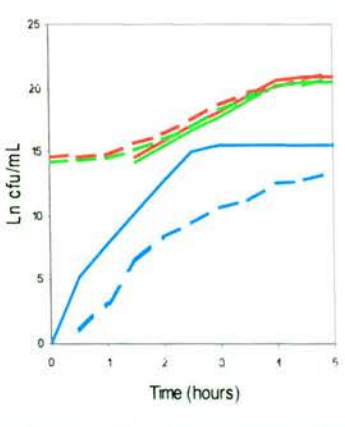
23



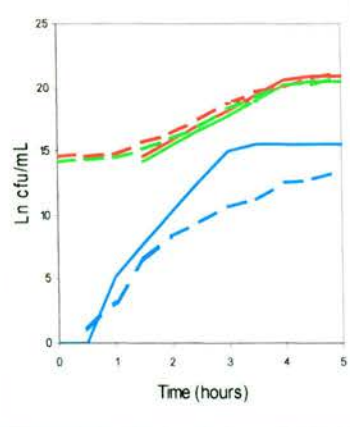
24



25



26



27